Hrudayanath Thatoi
Sonali Mohapatra
Swagat Kumar Das *Editors*

Bioprospecting of Enzymes in Industry, Healthcare and Sustainable Environment

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Hrudayanath Thatoi • Sonali Mohapatra •
Swagat Kumar Das
Editors

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Preface

The concept of enzymes or more appropriately natural catalysts is an area of interest for researchers for over a century. With the advent of recent technologies in the field of molecular biology, our knowledge base has developed even more in understanding the three-dimensional structures and the systematical modification of enzymes for their wider applications. The profound knowledge of the physiognomies of these enzymes has further extended the use of these workhorses in the fast pacing industrial sectors for production of value added products. In addition to bioprospecting, many groups are moving forward with the manipulation of enzymes for their applications in healthcare management and environmental-related issues. Although the industrial use of enzymes started with the use of pancreatic trypsin in 1913 by the German chemist Otto Röhm in textile industries, the enzymes have completely replaced conventional chemical degradation processes in practically every industrial sector. The usage of enzymes in industrial sector has been growing very fast with exploration of new applications. Thus, there is an urgent need to update the information on progress of enzyme technology for the scientific community and industrial personnel for the progress of knowledge and application of technological know-how for the benefit of mankind.

The book consists of 19 distinctive chapters contributed by eminent experts involved in research in the frontier areas of fermentation and enzyme technology, pharmaceutical, bioremediation, agricultural, textile, food and nutraceuticals with a focus on industrial application. With a comprehensive overview, this book has strived to focus on the recent information on trending applications of enzymes in bioremediation, agriculture, nutraceuticals and pharmaceutical industry along with biomass conversion for production of value added products. New concepts of enzymes in nano-technology, healthcare and nutraceuticals are well documented. The significance of important enzymes in textile industries and the unique role of thermostable enzymes in biofuel production are also presented. Experts from academia and industry articulate the cutting-edge technologies in the field of enzyme production and their application in different sectors of the industries. The editors are thankful to the distinguished authors for contributing their excellent piece of work

and experience gained in their respective fields in the form of chapters in the present book. This edited book will be useful not only for general students and academicians but also for researchers and industrial persons working in the field of bioprocess, fermentation, and enzyme technology.

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About the Editors

Hrudayanath Thatoi is a Professor in the Department of Biotechnology, North Orissa University, Odisha, India. He has around 15 years of teaching and 25 years of research experience. His research interests are microbial biotechnology, biofuel production, bioremediation, biodiversity conservation, mangrove biology, medicinal plants and bioactivity studies, ethnopharmacology, etc. Prof. Thatoi obtained his M. Phil and Ph.D. from Utkal University, Odisha, India, and his research work was based on N_2 fixation in legume plants under dual inoculation of Rhizobium and VAM fungi and contributed significantly towards the development of technology for mine waste reclamation in iron and chromite mine soils. He has handled many research projects of state government and central government organisations like University Grant Commission-Department of Atomic Energy, Govt. of India, Department of Science and Technology, Govt. of Odisha, Department of Forest, Govt. of Odisha, etc. Prof. Thatoi has published more than 200 research papers in various national and international reputed journals and around 30 book chapters. He has also authored around 15 books by different notable publishers. So far 15 students have already obtained Ph.D. under his guidance. Prof. Thatoi has contributed immensely to the field of microbiology and biotechnology throughout his research and teaching career.

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Application of Enzymes in Bioremediation of Contaminated Hydrosphere and Soil Environment

1

Sanchita Gupta, Lalit Dangi, Jayanta Kumar Patra, and Radha Rani

Dedication
The authors dedicate this chapter in fond remembrance of Sanchita Gupta, who left fingerprints of grace on our lives.

Abstract

Due to the ever-increasing global population, industrial sector is growing at a rapid rate which is leading to increase in discharge of hazardous and harmful substances in the environment. Enzymes play an important role in the management of environment by detoxifying or transforming harmful substances into useful products. A variety of enzymes have been isolated from bacteria, fungi, and plants having a wider application in degradation and/or transformation of toxic environmental pollutants. They have been used either in isolated or in bound form for the decontamination of water and soil contaminated with organic (pesticides, polyaromatic hydrocarbons, polycyclic biphenyls, etc.) and inorganic (heavy metals and radionuclides) pollutants. Enzymes that belong to oxidoreductase group detoxify the aromatic compounds through polymerization and co-polymerization with other substrates. This group includes oxygenases,

Sanchita Gupta was deceased at the time of publication.

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microbial laccases, and peroxidases. Apart from the aromatic compounds, heavy metals can also accumulate in the environment that leads to serious health problem because of their lyophilic, persistent, and toxic nature. For example, hexavalent species of chromium is more toxic than its trivalent species. Chromate reductase can convert the Cr^{6+} to Cr^{3+} which is more insoluble in water. This chapter presents an overview of application of different enzymes in waste water treatment and remediation of contaminated soil, sludge, and water.

Keywords

Enzymes · Bioremediation · Pollutants · Soil · Water · Environment

1.1 Introduction

Environmental pollution is among the major problems encountered in this century. As the human population grows, more resources are needed to support their life. Since the beginning of industrial revolution various industrial setup, agricultural activities and huge amount of waste generation are posing a burden of pollutants on this earth. Untreated sewage and industrial waste, landfills of dumped wastes, chemical fertilizers, and pesticides all are finally contaminating and polluting our water and land resources (Emenike et al. 2018). Pollutants can be classified on the basis of chemical nature like organic pollutants, inorganic pollutants, and radioactive pollutants. Organic pollutants include pesticides, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), benzene, toluene, ethylebenzene and xylene (BTEX), plastics, biopolymers, dyes, phenols, chlorophenols, etc. Pesticides include herbicide, insecticide, weedicide, rodenticide, etc. (Alcalde et al. 2006). The adverse effects of these chemicals on our environment came right from their production till their final application on agricultural fields and even after its usage as surface runoff. As most of them are nonbiodegradable and lipophilic, they accumulate in the body of organism and cause biomagnification, which is the significant rise of a substance in the body of organism from lower trophic level to higher trophic level. Besides agricultural fields, the sources of organic pollutants are effluents coming from different chemical industries such as leather, pulp and paper, and paints and petrochemical industries. Inorganic pollutants include cyanides and heavy metals like cadmium, mercury, arsenic, chromium, lead, copper, etc. (Wuana and Okieimen 2011). Sources of these inorganic pollutants include various anthropogenic activities such as industrial, agricultural, mining and metal processing, and natural soil weathering to some extent (Ayangbenro and Babalola 2017; Kushwaha et al. 2018). These pollutants can cause severe damage to human, animal and plant health. They can disrupt the biological system of plants and animals as well as human, and many of them have potential carcinogenic and teratogenic effects (Table 1.1).

As the increase of contamination of our natural environment poses severe negative impacts, it is necessary to detoxify and provide an effective solution to this

Table 1.1 Various pollutants and their harmful effects

SN	Pollutants	Harmful effects	References
1.	Organochlorine pesticides like DDT	Endocrine functionality disruption, neurodevelopmental defects in children, disruption of normal metabolism	Nunes and Malmlöf (2018)
2.	Arsenic	Skin cancer, dermatitis, black foot disease, brain damage, lung cancer	Ayangbenro and Babalola (2017)
3.	Cadmium	Itai-itai, bone damage, endocrine disruption, respiratory problem, and kidney damage	Ayangbenro and Babalola (2017)
4.	Chromium	Skin and respiratory irritation, lung cancer, renal failure	Garcia-Arellano et al. (2004)
5.	Mercury	Minimata disease, weakening of bone, memory loss, kidney damage, reduced fertility	Emenike et al. (2018)
6.	Lead	Brain damage, reduced intelligence in children, kidney damage, reduced fertility	Ayangbenro and Babalola (2017)

problem. During the last decade, new technologies in chemical, physical, and biological processes have been utilized to detoxify the soil and water environment. Bioremediation and phytoremediation are new approaches to abate the pollution problem. In both the strategies, enzymes are utilized to degrade the target contaminant. A large number of bacterial, fungal, and plant enzymes are found to be useful in degrading some toxic organic chemicals such as pesticides and in remediation of inorganic pollutants like heavy metals. Enzymes have several beneficial characteristics, like they can work in multiple environments, can readily be used with different substrates, can act intracellularly (inside the originating cell), extracellularly (in both presence or absence of originating cell), free as soluble in solution (catalysis will be homogenous), and immobilized attached to a solid matrix (catalysis will be heterogenous).

1.2 Bioremediation

Many physical and chemical treatments fail to remediate the pollution problem because of the production of residual after treatment and cost associated with it. These technologies utilize physico-chemical methods like soil vapor extraction, air sparging, chemical oxidation, and reduction. Organically contaminated soil by physical treatment is limited to the removal of volatile organic compounds (VOCs) only. A simple soil vapor extraction treatment become much more difficult and expensive when combined with control measures of air emissions. In chemical and irradiation method strong oxidants like H_2O_2 and ultraviolet (UV) light are required, respectively, which lead to the production of again chemical by-products (Fox 1996). On the other hand, bioremediation approach offers a great advantage with less/or no equipment, no residual products and more cost efficient. Bioremediation is

a process by which organic and inorganic wastes are biologically degraded. It utilizes naturally occurring or modified microorganisms like bacteria, fungi, yeast, and/or their products like enzymes to transform hazardous substances to a less or non-hazardous substances.

Bioremediation involves biodegradation (breakdown of complex organic chemicals into simpler forms), bioventing (injection of gas into subsurface to enhance the degradation process), bio-augmentation (introduction of microorganisms in contaminated area to facilitate the degradation process. Bioaccumulation (accumulation of organic or inorganic contaminants in an organism), biosorption (removal of substance through adsorption from a solution by biological material), biotransformation (transformation of xenobiotic substance in an organism's body via enzymes), and phytoremediation (use of plants for degrading pollutants like phytoextraction, rhizodegradation, phytovolatalization) (Kushwaha et al. 2018). The bioremediation technology is determined by several factors like site conditions, substrate-specific indigenous microorganisms, and amount and chemical toxicity of contaminants. Bioremediation strategies can be applied ex situ and in situ. In an in situ bioremediation strategy, contaminated water or soil is treated in its original site and ex situ remediation requires transfer of contaminated soil or water away from its original site to some other place where the treatment would be done.

1.3 Significance of Enzymes over Whole Microorganisms in Bioremediation

Bioremediation process depends on many factors like bioavailability of pollutants, optimum pH, temperature, presence of electron acceptors for aerobic and anaerobic conditions, nutrient availability, enzyme specificity, etc. As it is successful only when environmental conditions favor microbial proliferation and activity, environmental conditions are often manipulated to facilitate the degradation process. Both microorganisms and plants depend on enzymes required for transformation of polluting substances. Enzymatic remediation is a new approach toward the abatement of pollution. Enzymes have many advantages over microorganism used for degradation of pollutants. They are the main key component of all the transformation occurring in an organism. They may have either narrow or broad substrate specificity; therefore they can be applied to a large variety of pollutants (Aitken 1993). Substrate specificity of an enzyme is an ability to select exact chemical molecule from a mixture of similar components. A substrate specific enzyme recognizes its substrate through structural and conformational complimentarily between enzyme and substrate. Another advantage is that enzymes are not inhibited by inhibitors of microbial metabolism. Extreme pH and temperature, low nutrient availability, toxins, predators, and mixture of pollutants cause inactivation of microbial activity, but enzymes can carry out their actions under these harsh conditions which limit microbial activity. Enzymes are also effective at low pollutant concentration and viably catalyze reactions in the presence of microbial antagonists. They are more mobile than microorganisms due to their smaller size. Immobilized enzymes

(enzymes attached to a solid surface) are more feasible and economically viable because of their chemical stability and repeated use (Fan and Krishnamurthy 1995). All these properties make enzymatic remediation more suitable than microbial remediation.

1.4 What Are Enzymes?

Enzymes are biocatalysts that enhance the rate of metabolic reactions without themselves being changed. They do so by lowering the activation energy of a reaction. Enzymes are basically proteins. An enzyme binds to a substrate by its active site and transforms the substrate into product. Once the product is made, it is released from the active site, and the enzyme is now available for converting another molecule of substrate into product. Generally enzymes are of two type—simple enzymes, which consists entirely of amino acids, and conjugated enzymes, which contain a non-protein component, a cofactor, or a coenzyme along with a protein component.

1.4.1 Classification of Enzyme

Nomenclature committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) provided a systematic naming system for enzymes. According to the type of chemical reaction, they catalyze, and enzymes are named and categorized under six major classes as described below:

1. *Oxidoreductases*: They catalyze oxidation-reduction reactions. These include oxidases, oxygenases, dehydrogenases, reductases, peroxidases, and hydroxylases.
2. *Transferases*: They facilitate the transfer of chemical groups from one molecule to another. Examples of such groups involve amino (NH_2), carboxyl (CO), carbonyl, methyl (CH_3), phosphoryl, and acyl (RC=O). Some common examples are transaminases or amino transferases, transmethylases or methyl transferases, etc.
3. *Hydrolases*: They catalyze hydrolytic reactions that involve addition of water molecule to break bonds. The hydrolases include the esterases, phosphatases, etc.
4. *Lyases*: They catalyze reactions in which chemical bonds are broken down, other than hydrolysis and oxidation, to form a new double bond or a ring structure. They include decarboxylases and synthases.
5. *Isomerases*: They catalyze several types of intramolecular rearrangements and yield isomeric forms.
6. *Ligases*: They catalyze reactions that form C–S, C–C, C–O, and C–N bonds. For these reactions, energy is always provided by ATP hydrolysis (Gurung et al. 2013).

1.5 Microbial Enzymes and Their Application in Environmental Management

1.5.1 Oxidoreductases (EC 1)

Oxidoreductase comprises the large class of enzyme that catalyzes biological oxidation and reduction reactions via transfer of electrons from donor which is also called as reductant to recipient which is an oxidant molecule. It generally uses a nicotinamide adenine dinucleotide phosphate (NADP) or nicotinamide adenine dinucleotide (NAD^+) as cofactors. Oxidoreductase enzymes play a significant and diversified role in transformation of pollutants. During the previous years, there have been a number of significant new developments in the application of oxidoreductase enzymes, which includes oxidation of steroids and pharmaceuticals, oxidative degradation of pollutants, oxidation of hydrocarbons, and constructions of biosensors for a variety of clinical and analytical applications (May 1999) (Table 1.2).

1.5.1.1 Oxygenases (EC 1.13)

Oxygenases come under oxidoreductases class of enzymes. They play an important role in the oxidation of reduced substrates by transferring oxygen molecule via FAD/NADH/NADPH as a cofactor. There are two types of oxygenases that introduce either one or two oxygen atom(s) for oxygenation; they are monooxygenases and dioxygenases. By incorporating oxygen atom/molecule, a substrate tends to become more reactive and more water soluble, and also it causes opening of aromatic rings. The oxygenase enzymes are active against various compounds including aromatic, aliphatic, and halogenated organic compounds.

1.5.1.1.1 Monooxygenases (EC 1.14.13)

Monooxygenases introduce only one oxygen atom to the substrate. They act as catalyst in bioremediation process because of their high stereospecificity on a variety of target pollutants. They bring about dehalogenation, denitrification, desulfurization, hydroxylation, ammonification, transformation, and degradation of various aliphatic and aromatic compounds. Monooxygenases are grouped into two categories: flavin-dependent monooxygenases and P450 monooxygenases. Flavin-dependent monooxygenase has flavin as prosthetic group and requires NADP or NADPH as coenzyme. P450 monooxygenases are heme containing oxygenases and found in both prokaryotic and eukaryotic organisms (Kües 2015). Bacterial P450 monooxygenase CYP102 isolated from *Bacillus megaterium* BM3 is found to be able to hydroxylate a variety of alkanes. Some monooxygenases do not require cofactor for their activities. They are tetracenomycin F1 monooxygenase (TcmH) isolated from *Streptomyces glaucescens*, quinol monooxygenase (YgiN) from *E. coli* (Arora et al. 2010).

Phenol is an aromatic compound having wide application in chemical industries for the production of bisphenol A (BPA). It has also application in perfumeries, paint factories, plastic manufacturing, pharmaceutical industries, etc. For the degradation of mono aromatic compounds like phenol, initially phenol is oxidized to catechol by

Table 1.2 Enzymes and their environmental application

SN	Enzymes	Sources	Target pollutants	References
1	Monooxygenases	*Bacillus megaterium, Streptomyces glaucescens, E. coli*	Aromatic (chlorinated phenolic) and aliphatic compounds, etc.	Arora et al. (2010)
2	Dioxygenases	*Bacillus stearothermophilus, Pseudomonas pseudoalcaligenes, Comamonas testosteroni*	Cyclic aliphatic compounds and aromatic compounds like benzene, toluene, xylene, their derivatives like PCBs, etc.	Cowan and Fernandez-Lafuente (2011), Duran and Esposito (2000)
3	Laccases	*Phanerochaete chrysosporium, Lenzites betulina, Theiophora terrestris*, and white-rot fungi such as *Pleurotus ostreatus*, *Phlebia radiate*, and *Trametes versicolor*	Lignin polymer, polyphenol methoxy-substituted monophenols, PAHs, aromatic amines, etc.	Christian et al. (2005)
4	Peroxidases			
	(a) Lignin peroxidase	*Phanerochaete chrysosporium* *Trametes gallica* *Flavobacterium* sp. *Pseudomonas aeruginosa*	Lignin, PAHs, dyes	Karigar and Rao (2011)
	(b) Manganese peroxidase	*P. Chrysosporium* *Panus tigrinus* *Agaricus bisporus* *Bjerkandera* sp. BOS55	Lignin, PAHs (anthracene), milled wood, humic substances, dyes, amines	Hofrichter et al. (2010)
	(c) Versatile peroxidase	*Pleurotus, Bjerkandera* *Lepista, Trametes*	Lignin, dyes, pesticides	Asgher et al. (2008), Kadri et al. (2017)
5	Alkane hydroxylase	*Alcanivorax borkumensis, Methylococcus capsulatus, Pseudomonas, Methylomonas*, and *Methylobacter species.*	Linear chain aliphatic hydrocarbons	Piccolo et al. (2010)
6	Metal reductases	*P. Putida, E. coli, Bacillus megaterium*	Heavy metals like Cr^{6+}, Hg^{2+}, radionuclide like uranyl	Barak et al. (2006)
7	Nitroreductases	*Enterobacter cloacae, Staphylococcus aureus, E. coli*	Nitrocompounds like TNT	Hannink et al. (2007)

(continued)

Table 1.2 (continued)

SN	Enzymes	Sources	Target pollutants	References
8	Glutathione transferases	*Proteus vulgaris, P. mirabilis, Arabidopsis thaliana, Oryza sativa*	Herbicides like atrazine	Allocati et al. (2009), Hayes et al. (2005)
9	Cellulases	*Aspergillus niger*, *Acinetobacter junii*, some Actinomycetes such as *Cellulomonas fimi*	Cellulosic substances	Kuhad et al. (2011)
10	Proteases	*Bacillus cereus, B. megaterium, B. subtilis, P. aeruginosa*, etc.	Proteinaceous pollutants	Karigar and Rao (2011)
11	Lipases	*Achromobacter, Alcaligenes, Arthrobacter, B. burkholderia, Chromobacterium, Corynebacterium*, and *Pseudomonas species, Candida rugosa*	Triacylglycerol in fats and oil	Gurung et al. (2013)
12	Parathion hydrolases	*Pseudomonas diminuta*	Organophosphorus pesticides like methyl parathion	Serdar and Gibson (1985)
13	Alkyl sulfatases	*Pseudomonas* C12B	Detergents and surfactants, alkyl sulfates, alkyl ethoxy sulfates, aryl sulfonates	Dec and Bollag (2001)
14	Phosphatases	*Aspergillus nidulans, S. cerevisiae, Penicillium* sp.	Heavy metals and radionuclides	Lee et al. (2015), Pawar and Thaker (2009)
15	Cyanide hydratases	*Fusarium oxysporum, Pseudomonas teres, Aspergillus Niger, Gloeocercospora sorghi*	Cyanide containing effluents in the form of HCN, KCN, and R-CN	Martínková et al. (2015), Rinágelová et al. (2014)
16	Cyanide dihydratases	*Alcaligenes xylosoxidans*, subspecies *denitrificans, Bacillus pumilus, Pseudomonas stutzeri*	Cyanide compounds like HCN and R-CN	Martínková et al. (2015), Rinágelová et al. (2014)

phenol 2-monooxygenase (EC 1.14.13.7) (Comte et al. 2013). Catechol is further degraded by two different pathways. In one pathway that is brought about by ortho-cleavage, the aromatic ring is cleaved between the two hydroxyl groups by the

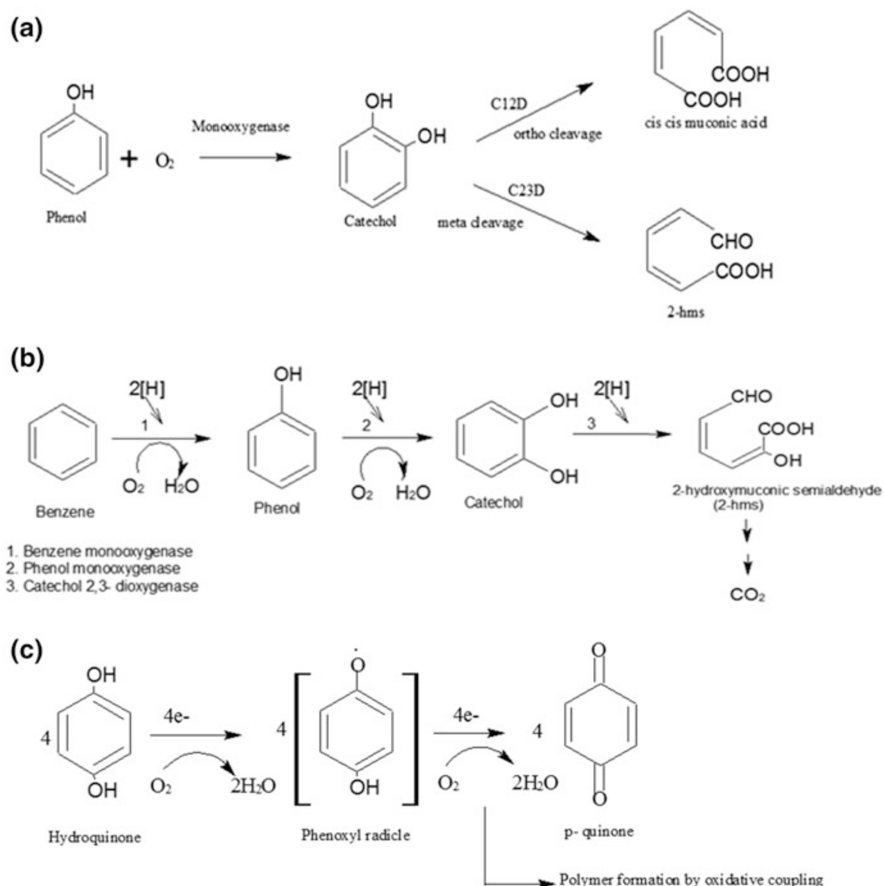


Fig.1.1 (**a**) Degradation of phenol by monooxygenase and dioxygenases (Modified from Arora et al. 2010 and Karigar and Rao 2011). (**b**) Degradation of benzene-ring activation by monooxgenases and ring cleavage by catechol 2,3-dioxygenase (Modified from Karigar and Rao 2011). (**c**) Oxidation of phenolic compound by laccase (Modified from Karigar and Rao 2011). (**d**) (**i**) The catalytic cycle of MnP, (**ii**) Degradation pathway of polycyclic aromatic hydrocarbon (Modified from Baborová et al. 2006; Eibes et al. 2006). *MnP* manganese peroxidases, *R* substrate. (**e**) Reduction of nitrocompounds by type I nitroreductases. The prosthetic group FMN is reduced with two electron transfer by NADPH and then R-NO_2 reduce to R-NH_2, R-NHOH by reduced FMN. *Enz* flavin-containing reductase, *FMN* flavin mononucleotide. (**f**) Biochemical pathway for the degradation of TNT. (Modified from Rylott et al. 2011)

catechol 1,2-dioxygenase [EC 1.13.11.1 (C12D)], leading to the formation of cis,cis-muconic acid. In the second pathway, meta cleavage occurs next to the two hydroxyl groups by catechol 2,3-dioxygenase [EC 1.13.11.2 (C23D)] and leads to the production of 2-hydroxymuconic semialdehyde (2-hms) (Fig. 1.1a).

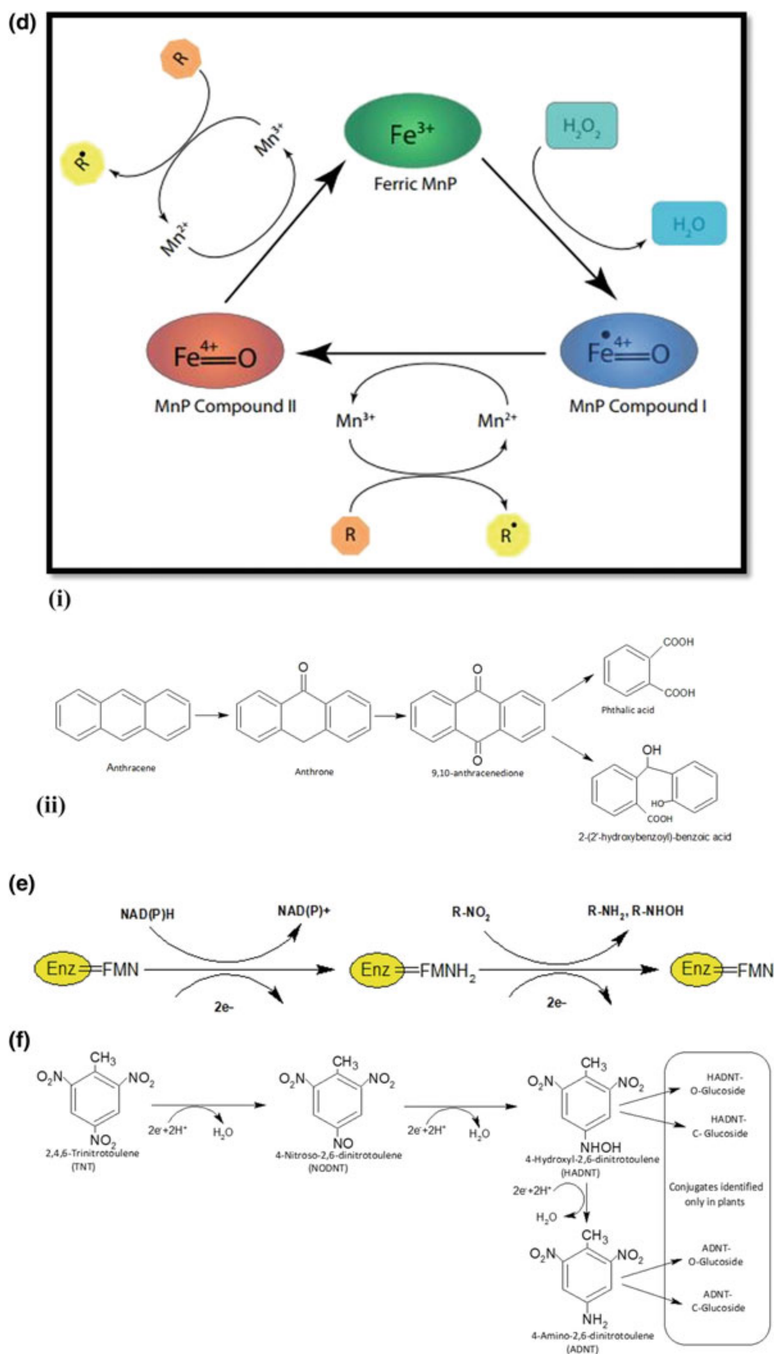


Fig.1.1 (continued)

1.5.1.1.2 Dioxygenases (EC 1.13.11)

Dioxygenase enzymes are oxidative enzymes. From prokaryotic to eukaryotic microorganism, all have evolved dioxygenase enzymatic activity by incorporating molecular oxygen into the substrate. It has been utilized for various metabolic pathways. The use of dioxygenases as oxidant is widespread from the generation of adenosine triphosphate (ATP) to the degradation of xenobiotics. Dioxygenases oxidize aromatic hydrocarbons (HCs) and therefore have applications in environmental remediation. Aromatic HCs have benzene-like structure; these compounds are more stable, as compared to other cyclic compounds, because of sharing of delocalized electrons by pi bonds as in benzene structure.

BTEX compounds are comparatively more mobile and water miscible. The two main steps involved in the degradation of aromatic compounds are (1) activation of the ring and (2) cleavage of the ring. Ring activation is achieved by the introduction of an oxygen molecule into the aromatic ring, which leads to dihydroxylation of aromatic nucleus. Dioxygenase incorporates oxygen molecule to form a dihydrodiol (Fig. 1.1b).

1.5.1.2 Laccases (EC 1.10.3.2)

Laccases (benzenediol oxygen oxidoreductases) are copper-containing phenol oxidase enzymes, which can oxidize electron-rich phenolic and non-phenolic substrate, subsequently reducing oxygen to water molecule (Duran and Esposito 2000). The radical undergoes spontaneous reaction beginning from the incorporation of an oxygen molecule, bond cleavage, and then finally the degradation of lignin polymer. Laccases are involved in lignin biosynthesis as well as its degradation, pigment formation, plant pathogenesis, iron metabolism, and kernel browning process (Jurado et al. 2011). Laccase enzymes are widely distributed in bacteria, fungi, insects, and higher plants. The most studied groups of enzymes are from the genera Ascomycetes, Basidiomycetes, and Deuteromycetes and cellulolytic fungi species including white rot fungi *Trametes versicolor, T. hirsuta, Pleurotus ostreatus, Phlebia radiata, Phanerochaete chrysosporium*, *Theiophora terrestris*, and *Lenzites betulina*. Bacterial laccases are found to be more stable at relatively high pH and temperature, and unlike fungal laccases, they are extracellular, such as *Azospirillum lipoferum* and *Bacillus subtilis*. The lignolytic enzymatic function of basidiomycetous fungi can be beneficiary for the degradation of organopollutants because these enzymes are evolved to degrade relatively water insoluble pollutants and generally lead to complete removal of the target pollutants due to the generation of free radicals (Christian et al. 2005). Lignin degrading enzymes are nonspecific and non-stereoselective. They act on a wide range and complex mixture of pollutants such as phenol, polyphenol, aminophenol, polyamines, and methoxy-substituted monophenols and can also oxidize inorganic compounds like iodine and ferrocyanide ions.

Laccase can mediate the redox process by having four copper atoms in their active site and are grouped in three types according to their stereospecific and magnetic properties.

1. Type 1 or blue copper center of laccase is organized with one methionine, one cysteine and two histidine molecules. It is the main key component responsible for the oxidation of substrate and redox potential of laccase.
2. Type 2 or normal copper center is coordinated with two histidines and a water molecule. It is colorless.
3. Type 3 or coupled binuclear copper center coordinates with three histidine ligands.

One type 1 and two type 3 Cu atoms together form a trinuclear copper center, which reduces oxygen to water molecule. All four atoms of copper in the native form of laccase remain in oxidized (Cu^{2+}) state. Since laccase catalyzes oxidation of substrate, the transfer of $4e^-$ from four laccase substrates by the type 1 Cu center to the trinuclear Cu center represents one cycle of substrate oxidation, and oxygen is reduced as being a final electron acceptor (Christopher et al. 2014) (Fig. 1.1c).

The excess level of glucose and sucrose concentration can reduce the laccase production, because being a secondary metabolite, it is produced under growth-limiting factors. The optimum temperature range for laccase production varies in microbes and plants. Most studies show that 4.5–6.0 pH is suitable for enzyme production (Shraddha et al. 2011). Small halide, azide, fluoride, and cyanide ions can prevent the enzymatic activity of laccase. Some divalent metal ions such as Hg^{2+}, Mg^{2+}, Ca^{2+}, and Zn^{2+} can also have an inhibitory effect on laccase activity by causing conformational changes or modifying the amino acid residues in the laccase glycoprotein.

1.5.1.3 Peroxidases (EC 1.11.1)

Peroxidases are the class of enzyme that belongs to oxidoreductases. They are categorized into two types: Heme peroxidase and Non-heme peroxidase. The ferriprotoporphyrin IX present as a prosthetic group in heme peroxidases, while such prosthetic group absent in the non-heme peroxidases. These enzymes carry out the oxidation of different types of xenobiotic compounds by utilizing the hydrogen peroxide as electron acceptor (Karam and Nicell 1997). They are further subdivided into two groups as animal and non-animal origin (bacterial, fungal, plants). The non-animal heme peroxidases are of three types (Karigar and Rao 2011; Abdullah et al. 2017):

1. Type 1, the intracellular peroxidases, includes ascorbate peroxidase (EC 1.11.1.11), which catalyzes the removal of hydrogen peroxide in higher plants; cytochrome c peroxidase (EC 1.11.1.5), it provides protection against toxic peroxides and catalase-peroxidases (EC 1.11.1.6) from bacteria, having catalytic properties of both peroxidase and catalase. Catalase-peroxidase is responsible for protecting the cells under oxidative stress.
2. Type 2 includes extracellular peroxidases: manganese peroxidases (EC 1.11.1.13) and lignin peroxidases (EC 1.11.1.14). These types of enzymes are glycoproteins in nature with two calcium-binding sites and four disulfide bridges in heme cavity. They are secreted extracellularly by fungi and participate

in the degradation of lignin, textile dyes, and other xenobiotics. Manganese peroxidases utilize the Mn^{2+} as reducing substrate.

3. Type 3 consists of the secretory plant peroxidases from soybean, barley, or horseradish, responsible for hydrogen peroxide removal, ethylene biosynthesis, biosynthesis of the cell wall, and oxidation of xenobiotic compounds. Like Class II, they are monomeric glycoprotein in nature, but differ in the placement of disulfide bridge.

   Mainly, the peroxidases of non-animal origin are used for the bioremediation of xenobiotic compounds. In this chapter, we focus on LiPs, MnPs, and VPs. These enzymes play important role in the wastewater treatment, medical kit development, food industry, decolorization of dyes from textile industries, polycyclic aromatic hydrocarbons degradation, and detoxification of phenolic contaminants among others in the environment.

1.5.1.3.1 Lignin Peroxidases [LiPs, EC 1.11.1.14]

As the name indicates, these enzymes mainly carry out the oxidation of lignin which is one of the main components of plant biomass in our environment. It is a glycoprotein of 38–46 kDa having 1 mole of iron protoporphyrin IX per mole of protein. They carry out the oxidation of xenobiotic compounds even in the absence of a mediator, because of its high redox potential. Lignin, which is formed by polymerization of p-hydroxyphenyl, guaiacyl, syringyl monomer via heterocyclic linkages, biphenyl bonds, and β-aryl ether linkages, offers the rigid structure to the plants and also protects the cellulose and hemicellulose from hydrolysis. It is a recalcitrant aromatic biopolymer, largely produced as a residue in industrial cellulosic ethanol plants, paper, and pulp industry. LiPs are also used for the decolorization of textile-based dye effluent, degradation, or biotransformation of polycyclic aromatic hydrocarbons (PAHs) and other xenobiotic compounds. Lignin peroxidases of both origins, bacterial and fungal, are capable of the degradation of lignin via oxidation (Behbahani et al. 2016; De Gonzalo et al. 2016). Lignin peroxidases from Basidiomycetes family have been well studied. They are of two types: white-rot fungi and brown-rot fungi. Lignin peroxidases, especially from white-rot fungus, are responsible for the degradation of plant biomass and other xenobiotics. The white-rot fungi producing the lignin peroxidase are *Phanerochaete chrysosporium, Phlebia radiata, Bjerkandera* spp., *Coriolus versicolor, Trametes gallica, Pycnoporus cinnabarinus,* and others (Hofrichter et al. 2010). The variety of bacteria produces LiP such as *Flavobacterium, Pseudomonas aeruginosa, Serratia marcescens, Xanthomonas, Arthrobacter, and Nocardia*.

The catalysis of substrate by LiP carries out in following steps (Kudanga et al. 2012):

Step 1: $\mathbf{Enz = Fe^{3+} + H_2O_2 \rightarrow Enz = [Fe^{4+} = O]R'_{COMPOUND\ I} + H_2O}$

In this step, the enzyme itself gets oxidized to Compound I by H_2O_2. Compound I is a two-electron-deficient intermediate that comprises the Fe^{4+} oxoferryl center and a porphyrin-based cation radical (R′).

Step 2: Enz = $\mathbf{[Fe^{4+} = O]R' + Substrate \rightarrow Enz = [Fe^{4+} = O]R_{COMPOUND\ II} + Oxidized\ substrate}$

In the second step, by one electron, Compound I oxidizes an organic substrate and forms a more reduced intermediate Compound II.

Step 3: Enz = $\mathbf{[Fe^{4+} = O]R_{COMPOUND\ II} + Substrate \rightarrow Enz = Fe^{3+} + H_2O + Oxidized\ substrate}$

Compound II can oxidize the second substrate and returns the enzyme to the resting state.

1.5.1.3.2 Manganese Peroxidases [MnPs, EC 1.11.1.13]

MnPs (Mn(II): H_2O_2 oxidoreductase) are extracellular glycoproteins with molecular weight ranging between 40 and 50 kDa and are secreted in the multiple isoforms in a culture medium supplemented with Mn. Like LiPs, MnPs also possess prosthetic group as an iron protoporphyrin IX. The white-rot fungus secretes MnPs, while no bacteria, yeast, and mold are capable of producing this enzyme. The enzyme functions by oxidizing Mn^{2+} to Mn^{3+} by reducing H_2O_2. This oxidation results in the release of strong oxidizer Mn^{3+} from the enzyme surface, which is quite unstable in aqueous media. The white-rot fungus that is capable of producing the organic acids (oxalate, malate, glyoxylate) and chelate Mn^{3+} results in the formation of stable complex. The complexed Mn^{3+} ions act as a diffusible charge transfer mediator, which can oxidize the phenolic compounds such as natural and synthetic lignin, PAHs (anthracene, phenanthrene, and fluoranthene), milled wood, dyes, amines, humic substances, and other xenobiotic compounds. The white-rot fungus–producing MnPs are *P. chrysosporium*, *Panaus tigrinus*, *Agaricus bisporus, Nematoloma frowardii*, and *Bjerkandera* sp. (Asgher et al. 2008; Kadri et al. 2017).

The catalytic cycle is initiated when native ferric enzyme interacts with H_2O_2, results in the formation of Mn compound 1, which is a Fe^{4+}-oxo-porphyrin-radical complex. Afterwards, one molecule of water expelled, when the dioxygen is heterolytically cleaved. The porphyrin intermediate receives one electron when a monochelated Mn^{2+} gets oxidized to Mn^{3+} and forms Mn compound II. The native enzyme is generated from Compound II in a similar way through the transfer of one electron from Mn^{2+} to form Mn^{3+}, thereby leading to generation of second water molecule. The chelated Mn^{3+} ions generated carry out the oxidation of polycyclic aromatic hydrocarbons, various phenolic substrates, dyes, amines, as well as lignin model compounds, e.g., anthracene (a PAH) is made up of three fused benzene rings. The red dye alizarin and other dyes are produced using anthracene. The oxidation of polycyclic aromatic hydrocarbon carried out by MnP results in the formation of respective quinones. The first intermediate formed during degradation of anthracene is anthrone, followed by the production of 9,10-anthraquinone. As in the case of MnP from *Bjerkandera* sp. BOS55, more oxidation leads to the formation of phthalic acid. MnP also has a capability to cleave aromatic ring of polycyclic

aromatic hydrocarbon molecules and results in the formation of 2-(2-′-hydroxybenzoyl)-benzoic acid (Baborová et al. 2006; Eibes et al. 2006) (Fig. 1.1d).

1.5.1.3.3 Versatile Peroxidases [VPs, EC 1.11.1.16]

Versatile peroxidases, structurally LiP-like hybrid manganese peroxidase. This peroxidase comprises the substrate-specific properties of LiP and MnP fungal peroxidase families. It was obtained from plant roots and wood-colonizing white-rot basidiomycetes *Pleurotus eryngii* and also found in *Bjerkandera* sp. Due to its catalytic versatility, it carries out the oxidation of Mn^{2+} and also phenolic and non-phenolic aromatic compounds. Even high redox potential compounds such as Reactive Black 5 dye can also be oxidized in Mn-independent reactions. It has been suggested that VPs provide multiple binding sites for substrate due to its hybrid molecular structure. By the virtue of this property VPs is more superior and effective over both LiP and MnP, which are not able to degrade xenobiotics without the presence of veratryl alcohol and Mn^{2+}, respectively. That means due to dual catalytic properties, VPs are capable of oxidizing variety of (low and high redox potential) substrates including Mn^{2+}, dimethoxybenzenes, veratryl alcohol, substituted phenols and hydroquinones, and different types of reactive dyes (Hofrichter et al. 2010). Till date, only fungal species are able to produce VPs such as *Pleurotus, Bjerkandera, Lepista*, and also by *Panus* and *Trametes* (Ruiz-Duenas et al. 2009).

The catalytic cycle of VPs is similar to that of LiPs, in which ferric resting enzyme undergoes two electron oxidation which leads to the formation of compound I by reaction with H_2O_2. The compound I oxidizes a veratryl alcohol and results in the formation compound II that further oxidizes the second molecule of veratryl alcohol subsequently being reduced to the resting state forming free enzyme.

Pesticides are chemical compounds that are used to kill or inhibit pests including rodents, insects and unwanted plants (weeds). Only 5% of pesticides target the organisms and remaining part move to the water bodies and soil or air, gain entry in the food chain, and cause serious environmental and health problems. The carbon at the para position of chlorinated phenols (tri-, tetra-, and penta-chlorinated) undergo oxidative dehalogenation by this enzyme. Dichlorophen, which is penta-chlorophenol, transformed to quinones mediated by the versatile peroxidases. Initially, a free radical is produced and undergoes delocalization from the phenolic oxygen to para carbon position of the aromatic ring. After that, carbon cation has to be formed by second electron extraction. Then a para-benzoquinone is formed by the addition of hydrogen by nucleophilic attack.

1.5.1.4 Alkane Hydroxylases (1.14.14.3)

Alkanes are saturated linear HCs, basically made up of carbon and hydrogen, whose chain length can vary from 1 to 50 carbon atoms. The general formula for an alkane is C_nH_{2n+2}. Alkanes are mainly found in crude oil. As C–H bond in alkane is difficult to cleave, it is difficult to introduce hydroxyl group on an inactivated hydrocarbons. Alkane hydroxylases (AHs) also known as alkane monooxygenases activate the aliphatic chain by oxidation of one of the terminal methyl group to generate corresponding primary alcohol under aerobic condition. Most AHs tend to have wide substrate range and can be used to synthesize alcohols, aldehyde, carbonic

acids, and epoxides. Thus, alkane degraders are useful in oil-polluted environment (Piccolo et al. 2010; Van Beilen and Funhoff 2007).

1.5.1.5 Metal Reductases (EC 1.16)

Some oxidoreductases have promising role in transforming more toxic valence state of heavy metals into less or non-toxic valence state. These are chromate reductases, uranyl reductases, mercuric reductases, etc. Chromate is present in various industrial effluents mainly in leather-tanning industry, chrome plating, and pigment production processes. Chromate is highly water soluble, which leads to contaminate water resources. Bacterial enzymes like ChrR in *P. putida* and YieF in *E. coli* and can reduce chromate (Cr^{6+}) to (Cr^{3+}) valence state, which is less water soluble and also much less toxic. They are aerobic oxidoreductase associated with soluble proteins and requires NAD(P)H as an e^{-} donor. Enzymatic reaction of Cr^{6+} to Cr^{3+} involves transfer of electron from donor molecule like NAD(P)H to recipient Cr^{6+} and simultaneous generation of ROS (Garcia-Arellano et al. 2004; Barak et al. 2006). Some reductases are also associated with membrane like cytochrome c reductase, use chromate as terminal electron acceptor for generation of energy (Thatoi et al. 2014). Likewise, uranyl [U(VI)] is also water soluble and subjected to leach out in water resources and therefore poses a potential threat to environment, its reduced valence state U(IV) is insoluble. Mer A gene and Mer B found in *Bacillus megaterium* MB1 encodes for mercuric reductase (EC 1.16.1.1) and organomercurial lyase, respectively, these enzymes are found to be able to transform Hg^{2+} and methyl mercury to insoluble elemental mercury.

1.5.1.6 Nitroreductases (EC)

They are flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD)–dependent enzymes that carry out detoxification of nitrocompounds such as nitroaromatic and nitro-substituted compounds which are characterized by the presence of one or more nitro group, using NADH or NADPH as reducing agents. These enzymes are involved in the transformation of toxic nitro compounds to its derivatives with no toxic/mutagenic activity. The nitro group containing compounds such as nitropyrenes, nitrobenzenes, nitrofurans, and several others have wide range of applications in food additives, antimicrobial agents, pesticides, explosives, dyes, and other industrial processes. Due to their wide range of application, they are categorized as important group of pollutants in the environment. In the nitro group, high electronegativity of oxygen attracts nitrogen's electrons which result in the formation of partial negative and positive poles. The positive pole formed has great tendency to undergo reduction, as it tends to attract electrons. Thus, based on the reduction of nitro group by one or two electron transfer in the presence of oxygen, they have been grouped into two categories (Hannink et al. 2007).

Type I (oxygen-insensitive) nitroreductases: they are responsible for the reduction of nitro groups of nitrocompounds in two electron increments to form nitroso derivatives. These enzymes utilize the NAD(P)H as an electron donor and further hydroxylamine and amine are produced from unstable nitroso derivatives depending on the source and type of the enzyme (Fig. 1.1e).

Type II (oxygen-sensitive) nitroreductases: perform one electron reduction of nitro group in the presence of oxygen and produce a nitro anion radical. The transfer of electron to molecular oxygen from the nitro anion radical is formed which results in the generation of superoxide and regeneration of original nitroaromatic compounds. Thus, only anaerobic conditions favor the reduction of nitroaromatic compounds via two-electron transfers.

From above nitroreductases, type I nitroreductases are most important as they catalyze the reduction of a variety of nitrocompounds including nitrotoluenes, nitrobenzoate, nitrophenols, nitrobenzene, and nitrofurans via two electron transfer. The organisms able to produce nitroreductases are *Bacillus amyloliquefaciens, Bacillus subtilis, Enterobacter cloacae*, *Escherichia coli*, *Staphylococcus aureus, Salmonella typhimurium,* and *Klebsiella* sp. They are also produced by yeast such as *Saccharomyces cerevisiae* and also by plants such as *Catharanthus roseus, Phaseolus vulgaris, Myriophyllum aquaticum, Populus tremula, Panicum virgatum, Chrysopogon zizanioides,* and hybrid *Aspen* (*Populus tremula* × *tremuloides*). The bacterial nitroreductases have been considered for decontamination of soil with nitrocompounds and also related hazardous organonitro compounds. Some of the anaerobic bacterial nitroreductases catalyze the reduction of other compounds without nitro groups such as azo dyes. The azo dyes contain N=N bonds, which are cleaved by these enzymes to produce amines (Rafii et al. 2005). Recent studies reveal that they are used in phytoremediation in which transgenic plants expressing nitroreductases are employed for detoxification of contaminated hydrosphere and soil with carcinogenic compounds. As the bacterial species unable to produce high enough biomass to remove significant amounts of nitrocompounds, genetic engineering technology provides us a tool for expressing the bacterial enzyme in the plant which improves their ability for uptake and detoxification of xenobiotic compounds. The process of detoxification in the plants carried into three phases. In Phase I, where xenobiotic compounds lacking reactive group are activated by introducing functional groups (commonly hydroxyl, amino, and sulfhydryl). This activation enables Phase II, where conjugation occurs in which plant metabolites are conjugated to the activated xenobiotic. In Phase III, sequestration of conjugated xenobiotic occurs in plant organelles or structure (Rylott and Bruce 2009). For example, 2,4,6-trinitrotoulene (TNT) most widely used explosive by military contains three nitro group, and it is a potential mutagen and group C human carcinogen (Das et al. 2015). Thus, phytoremediation used for the removal of mutagenic explosives from the contaminated soil and water system. The enzyme expressed in the plants transforms TNT to hydroxylaminodinitrotoulene (HADNT) and aminodinitrotoulene (ADNT) by one or more nitro group's reduction of TNT via nitroso intermediate. This is reduction favored because nitro groups of TNT have electron withdrawing property, making the aromatic ring of TNT electron deficient. After transformation, glutathione S transferases (GST) and UDP-glycosyltransferase (UGT) families carry out the conjugation at either the 4-isomer or 2-isomer positions of HADNT or ADNT, forming either O-glucosidic or C-glucosidic bonds (Rylott et al. 2011) (Fig. 1.1f).

1.5.2 Transferases (EC 2)

1.5.2.1 Glutathione Transferases (EC 2.5.1.18)

Glutathione transferases (GSTs, EC 2.5.1.18) are group of enzymes found in aerobic bacteria along with plants, insects, fish, and mammals and play a vital role in variety of cellular processes such as biosynthesis of hormones, protection against oxidative and chemical stresses and detoxification of xenobiotic compounds. Till date, it has been identified that no anaerobic bacteria or archeabacteria is able to produce such enzyme (Allocati et al. 2009). They are soluble proteins composed of two polypeptide subunits and able to catalyze nucleophilic attack on nonpolar compounds containing electrophilic atom (C, N, and S) by tripeptide glutathione (GSH) (Hayes et al. 2005). These enzymes catalyze the glutathione-dependent transformation of toxic compounds including industrial pollutants, antibiotics, genotoxic carcinogen metabolites, herbicides, and insecticides. They are responsible for detoxification of xenobiotics by facilitating the conjugation reactions with reduced tripeptide glutathione and result in water-soluble products that are more readily excreted (Enayati et al. 2005).

In conjugation reaction, the residue at the active site of GST interacts with sulfhydryl group (-SH) of cysteine of GSH. This interaction results in the generation of active thiolate anion (GS-), that catalyzes the nucleophilic attack on the electrophilic center of non-polar compounds to form GS-Conjugate which leads to detoxification. The conjugate product is more water soluble and easily excreted than non-conjugated compound via export proteins (Labade et al. 2018). The general reaction is as follow: R-X + GSH $\rightarrow$ R-SG + XH, where R is xenobiotic and X is leaving group.

Each GST consists of two domains: N-terminal thioredoxin like domain specific for binding of GSH (G-site) and C-terminal domain composed of $\propto -$ helix provides the binding site for hydrophobic co-substrate (H-site). A short variable region of 5–10 residues is also between these two binding sites. The crystal structure reveals that G and H sites of the enzyme are mobile that leads to conformational changes on binding of the substrates (Dixon et al. 2010). On the basis of their localization in the cell, GSTs constitute three major superfamilies: the mitochondrial, the cytosolic GSTs (largest superfamily), and integral membrane protein the microsomal GSTs, now designated as membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEGs). The cytosolic GSTs are further classified on the basis of their sources (Oakley 2011). In this chapter, GSTs from aerobic bacteria and plants are of interest which play important role in the bioremediation of contaminated ground water and soil. The bacterial species showing GSTs activity are: *Proteus mirabilis, E.coli, Ochrobactrum anthropic, Burkholderia xenovorans, Synechococcus elongatus, Proteus vulgaris, Agrobacterium tumefaciens*, and many more (Allocati et al. 2009). Plants for detoxification of contaminated sites having GSTs are: *Oryza sativa, Arabidopsis thaliana, Triticum aestivum, Zea mays,* and *Glycine max* (Cummins et al. 2011). GSTs from different sources carry out the detoxification of xenobiotic compounds. For example, several classes of herbicides undergo detoxification process by GSTs. Atrazine, most widely used herbicide,

belongs to triazine class. In biodegradation of atrazine, the first step is catalyzed by GSTs. They carry out the removal of chlorine atom by forming atrazine–GSH conjugate. After dechlorination, hydroxylase enzymes remove isopropylamine, and ethylamine groups in a step-wise manner by dealkylation (Fig. 1.2a).

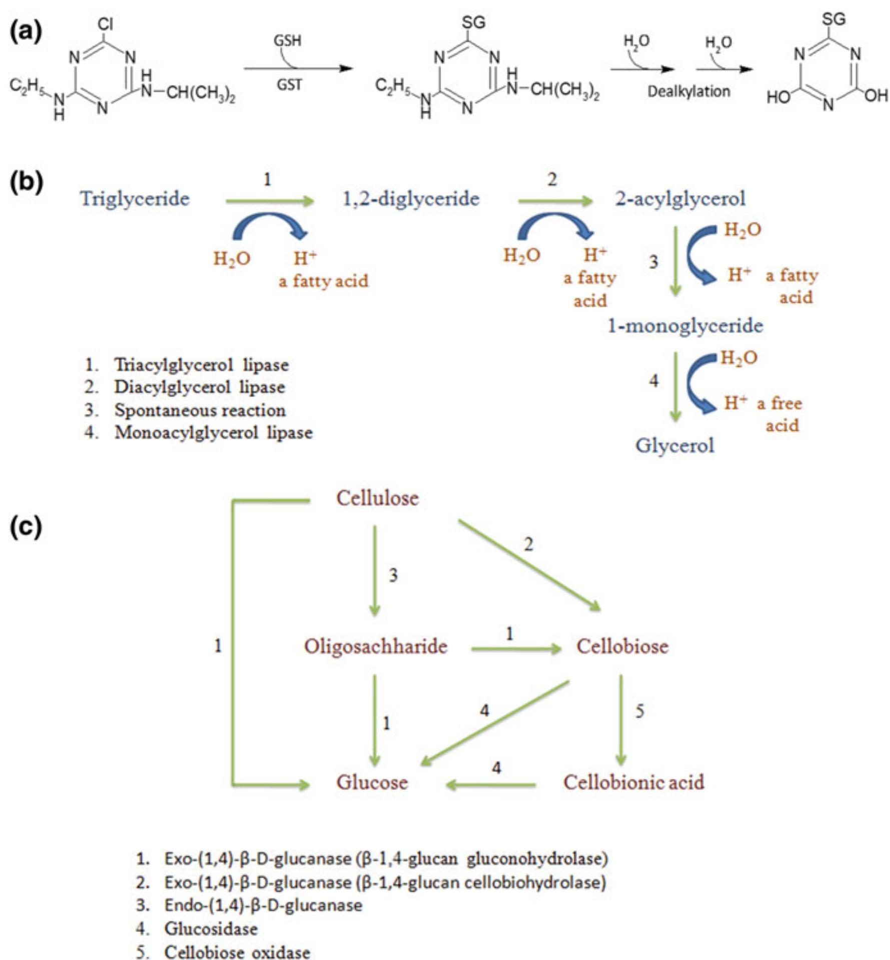
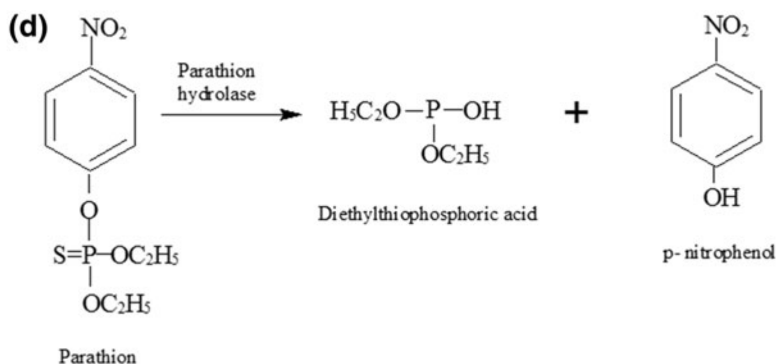


Fig. 1.2 (**a**) A proposed degradation pathway of atrazine by bacterial GSTs (Adapted from Allocati et al. 2009). (**b**) Transformation of a triglyceride into glycerol and free fatty acids. (**c**) Cellulose degradation pathway through various cellulase enzymes (Kuhad et al. 2011). (**d**) Parathion degradation through parathion hydrolase (Serdar and Gibson 1985). (**e**) Degradation pathway of sodium dodecyl sulfate (SDS). (Modified from Chaturvedi and Kumar 2010)



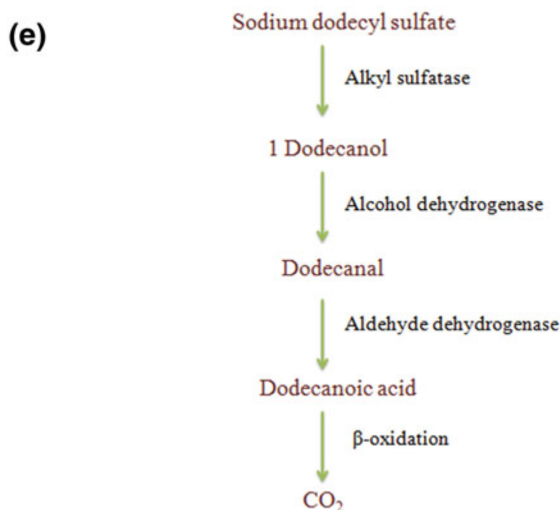


Fig. 1.2 (continued)

1.5.3 Hydrolases (EC 3)

1.5.3.1 Lipases (EC 3.1.1.3)

Lipases (triacylglycerol acylhydrolases) have wider applicability in the degradation of lipid waste. Lipase enzymes catalyze the hydrolysis of fats and oil into fatty acids and glycerol (Gurung et al. 2013). The triglyceride ester bonds are broken down in the presence of water and glycerol and fatty acids are produced as end products. This reaction occurs through transesterification in which the triglyceride gets converted into diglyceride. Then, diglyceride gets transformed into monoglyceride, which finally gets converted into glycerol (Okino-delgado et al. 2017) (Fig. 1.2b).

1.5.3.2 Cellulases (EC 3.2.1)

Cellulase enzymes carry out hydrolysis of cellulose. It is distributed in a variety of microorganisms like *Aspergillus niger*, *Acinetobacter junii*, some Actinomycetes such as *Cellulomonas fimi,* etc. Three types of cellulases are identified which are as follows:

1. Endo-(1,4)-β-D-glucanase (EC 3.2.1.4): It attacks on the end of the cellulose chain and release β-cellobiose.
2. Exo-(1,4)-β-D-glucanase (EC 3.2.1.91): It randomly attacks the internal O-glycosidic bond, which results in the formation of glucan chain of varying lengths.
3. β-Glucosidase (EC 3.2.1.21): It attacks β-cellobiose disaccharide and releases glucose moiety (Kuhad et al. 2011) (Fig. 1.2c).

The wastes originated from forests, agricultural fields, and agronomical industries contain enormous amount of unused cellulosic biomass causing extra spacious load to environment. Most of the time they are burnt which create another pollution problem. Nowadays, these wastes are processed by cellulase enzymes to produce valuable energy sources like bioethanol, etc.

1.5.3.3 Proteases (EC 3.4)

Protease belongs to a group of enzymes that hydrolyze peptide bonds. They are protein degrading enzymes that break polypeptide chains of protein into shorter fragments and eventually into their amino acid monomers (Karigar and Rao 2011). Proteases are broadly classified in two types: (1) endo-peptidases and (2) exo-peptidases. Proteases are also classified as acidic, alkaline, and neutral proteases based on their pH optima. Proteases have a wide range of application such as in leather, detergent, food, and pharmaceutical industries as well as in silver recovery and bioremediation strategies.

The largest application of protease is in laundry and leather industries, where they remove proteinaceous stains from clothes and serve as dehairing agent, respectively. Another area of application is in the management of industrial and household wastes, conventional methods of dehairing involve the use of lime and sodium sulfide as dehairing agents. But now it is replaced with proteases. Thus, enzyme-based dehairing reduces the chemical load on the environment. Bacterial proteases have potential application in the management of wastes from household and processing industries, also cleaning of hair-clogged pipelines containing hairs. Proteases can solubilize wastes from slaughter houses.

1.5.3.4 Parathion Hydrolases (EC 3.1.8)

Parathion hydrolases are useful in the degradation of organophosphorus (OP) compounds. Some OPs are highly toxic. Use of OPs in agriculture causes a serious concern over food safety and environment deterioration. Methyl parathion hydrolase [(MPH) EC 3.1.8.1] is an important OP hydrolase that exists in many bacterial species (Deng et al. 2015). MPH isolated from *Pseudomonas* sp. WBC-3 is

found to catalyze the degradation of methyl parathion, which generates a yellow-colored end product (Yang et al. 2008). *Pseudomonas diminuta* strain MG hydrolyses parathion (*o*,*o*-diethyl-*o*-*p*-nitrophenyl phosphorothioate) in an OP that is extremely toxic to diethylthiophosphoric acid and *p*-nitrophenol (PNP), which reduces its toxicity nearly 120-folds (Serdar and Gibson 1985) (Fig. 1.2d).

1.5.3.5 Sulfatases (EC 3.1.6)

These are enzymes of esterase class that hydrolyzes sulfate esters. They are important in the degradation of sulfated compounds and for cycling of sulfur in the environment (Chaturvedi and Kumar 2011). Three classes of sulfatases have been identified according to their substrate type and mechanism of action:

1. *Aryl sulfatase*: Aryl sulfatase is systematically known as aryl sulfate sulfohydrolase. It catalyzes the hydrolysis of phenol sulfate into phenol and sulfate.
2. *Fe^{2+}-dependent sulfatase*: It belongs to the Fe^{2+}-dependent dioxygenase group of enzymes which oxidatively break the bonds of the sulfate ester to form the corresponding aldehyde and inorganic sulfate. For the biocatalytic application, it is less important.
3. *Alkyl sulfatase*: It has potential application in bioremediation. These enzymes are able to degrade soil contaminated by detergents. Sulfate ester is cleaved by two methods. First is the cleavage of S–O ester bond, which releases an alcohol. Other is the addition of a water molecule in order to break C–O bond and release of an alcohol molecule from the sulfate ester (Toesch et al. 2014).

Degradation of sodium dodecyl sulfate (SDS) is initiated by primary alkyl sulfatase enzyme followed by the oxidation of the released alcohol and aldehyde by appropriate alcohol dehydrogenase and aldehyde dehydrogenase enzymes, respectively, and finally β-oxidation of subsequently liberated acid (Chaturvedi and Kumar 2010) (Fig. 1.2e).

1.5.3.6 Phosphatase (EC 3.1.3)

As the various industrial sectors such as nuclear power plant, mining, smelting, waste materials, and domestic contaminant lead to release of heavy metal and radionuclide pollutants in the environment. The bioaccumulation of heavy metals and radionuclides is a serious problem and major challenge of removal of desired metals and radionuclides from such wastes. Phosphatase is a hydrolase enzyme which plays an important role in the hydrolysis of organic phosphates and polyphosphates by cleaving the C–O–P ester bonds in the phosphate metabolism of the organism. The enzyme phosphatases catalyze the reaction in which insoluble metal-phosphate precipitate is formed which is responsible for removal of heavy metals and radionuclides from the environment. In this reaction, HPO_4^{2-} liberated from organic or inorganic phosphate source that precipitates stoichiometrically with heavy metals (M) to form $MHPO_4$ (Açıkel and Erşan 2010). Phosphatases also play a crucial role by releasing assimilable phosphate from various organic sources in supporting microbial nutrition (Nilgiriwala et al. 2009). There are two types of

phosphatase enzymes: acid phosphatases and alkaline phosphatases. Both the types of phosphatases carry out the same type of reaction, but at different pH range. Acid phosphatases (EC 3.1.3.2, orthophosphoric monoester phosphohydrolase, ACP) are found in many fungi, bacteria, plant, and animal species. They work under acid pH condition and specifically hydrolyzing the phosphate esters (Pawar and Thaker 2009). The organism producing ACPs are: *Aspergillus nidulans, Saccharomyces cerevisiae, Penicillium species, Aerobacter aerogenes, Escherichia coli, Burkholderia gladioli,* and *Rhizopus delemar* (Açıkel and Erşan 2010). Alkaline phosphatases (EC 3.1.3.1, APase) are hydrolase enzymes which efficiently carry out the hydrolysis of several mono- and diesters of phosphate at alkaline pH. The crystal structure at resolution 1.75 Å reveals that the active site consists of one Mg^{2+} ion which is important in structural stability and two Zn^{2+} that involves in catalysis reaction. Alkaline phosphatase requires divalent ions for their activity while EDTA acts a chelating agent and inhibits the activity. The organism producing APase: *Escherichia coli, Sphingomonas* sp. BSAR-1*, Arthrobacter* sp., *Citrobacter* sp. N14*, Bacillus* sp., *Aspergillus niger*, *Deinococcus radiodurans* R1, and *Pseudomonas aeruginosa* (Appukuttan et al. 2006). Phosphatase's catalytic sites have serine residue that gets phosphorylated, and then hydrolysis of the substrate (phosphoanhydride or phosphoester) is initiated. In the next step, inorganic phosphate is produced via hydrolysis of phosphoseryl intermediate, which can bind with metal and result in the formation metal-phosphate precipitate. This mechanism is responsible for removal of heavy metals (lead, nickel, chromium, and cadmium) and radionuclides (Uranium) from the soil and ground water as follows (Lee et al. 2015; Chaudhuri et al. 2013).

1.5.4 Lyases (EC 4)

1.5.4.1 Cyanide Hydratases (EC 4.2.1.66) and Cyanide Dihydratases (EC 3.5.5.1)

These enzymes belong to the class of nitrilases super family. Nitrilases enzymes are those that act on non-peptide C–N bonds. They have significant role in detoxification of hydrogen cyanide released from various industrial effluents such as mining industries and coal coking effluent. Cyanides also enter in the environment due to natural synthesis and also due to large-scale production of inorganic cyanide as CN- and HCN as well as organic cyanides as nitrilase, RCN (Rinágelová et al. 2014). CHT is an extracellular enzyme found in few filamentous fungi and bacteria, such as *Fusarium oxysporum, Pseudomonas teres, Aspergillus niger, Gloeocercospora sorghi,* etc. Cyanide hydratases (CHTs) hydrolyze hydrogen cyanide into formamide. CynD is produced by few bacterial genera like *Alcaligenes xylosoxidans* subspecies *denitrificans, Bacillus pumilus,* and *Pseudomonas stutzeri*. The reaction product of cyanide dihydratases (CynD) is formic acid and ammonia (Martínková et al. 2015) (Fig. 1.3).

Fig. 1.3 Degradation of hydrogen cyanide into formate and ammonia via cyanide hydratase and amidase enzymes. (Modified from Martínková et al. 2015)

1.6 Limitations

The application of extracellular and/or cell free enzymes in the remediation of polluted environment is an innovative technique. They offer some advantages over using whole microbes as decontaminating agent. However, several limitations restrict their practical application. In an enzyme-based treatment of contaminated soil or water the most important thing to be considered is its cost. Enzymes that are currently being explored are expensive due to the cost of their isolation, purification, and production. Additionally isolated enzymes demands for the following requirements:

1. A suitable enzyme must be identified in order to be specific toward the target pollutant.
2. Source of enzyme should be identified by selective screening process.
3. The enzyme producing microorganism must be cultured to extract the enzyme for cell free or immobilized action.
4. It should be suitable under operational condition.

Several pit-holes may hinder the catalytic efficiency of enzymatic remediation. In contaminated sites, many organic and/or inorganic chemicals are present; as a result, possible negative or positive effects on the catalytic activity of enzymes may occur.

1.7 Conclusion

This chapter has emphasized the capability of enzymes accelerated remediation of polluted soil and aquatic environment. As the amount of various organic pollutants is increasing in our environment, it is very obvious to utilize the potential impact of oxidoreductases like mono- and dioxygenases, peroxidases, and laccases activity on not easily degradable organic pollutants. The main goal of enzymatic remediation should be revitalizing soil health and fertility, detoxification of ground and surface water, and reutilization of wastewater. Therefore, understanding of an enzymatic pathway for decontamination needs to be known to apply the remediation strategy on polluted sites. Challenges that need to be overcome in the application of enzymatic degradation of contaminants include: (1) reduction in the production cost,

(2) improved recyclability, (3) ability to efficiently degrade phenolic and non-phenolic compounds, (4) stability of enzymes, and (5) no inhibitory effects on enzymes. More research needs to be done on applicability of an enzyme, so that it can provide a sustainable and eco-friendly solution for pollution.

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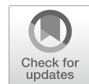

2 Bioremediation of Highly Toxic Hexavalent Chromium by Bacterial Chromate Reductases Family: A Structural and Functional Overview

Hrudayanath Thatoi and Manish Paul

Abstract

Chromium is a heavy metal with a vast range of industrial applications. However, extensive mining of chromite ore and its industrial application pose a serious threat to human health due to emission of toxic hexavalent chromium ions to the environment. Cr(VI) (chromate) is highly soluble and toxic ion which is a widespread environmental contaminant. Presently, several physicochemical methods are there for detoxification of Cr(VI) which are associated with high cost and typically cause secondary pollution. In this context, bacteria-mediated chromate reduction process is both economical and eco-friendly. Different chromate reductases have been identified from bacterial sources. According to sub-cellular localization, bacterial chromate reductase has been classified as cytoplasmic and membrane bound. Cytoplasmic chromate reductases are promising candidate for protein engineering and thus can be used in bioremediation program. The enzyme chromate reductase can catalyze its reduction mechanism in either aerobic or anaerobic condition. Chromate reductase–induced reduction of Cr(VI) to Cr(III) is mediated by the shifting of electrons from a nucleophilic cofactor like NAD(P)H to Cr(VI) and concomitant production of reactive oxygen species (ROS). Depending upon the steps present in the reduction process and the amount of ROS generation, two mechanism of action for the enzyme chromate reductase has been proposed known as Class I "tight" and Class II "semi-tight." This chapter highlights various classes of bacterial chromate reductases, their three-dimensional structure, functions, and prospective uses in the bioremediation of hexavalent chromium.

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Keywords

Bioremediation · Oxidoreductase · Chromate reductase · Quinone reductase · NADPH FMN reductase · Enzyme activity · Enzyme structure

2.1 Introduction

Chromium is a naturally found heavy metal and has various industrial purposes such as in metallurgical works, oil refinery, leather tanning, and production of cloth material, color, and steel as well as in the production of thermonuclear weapons. However, chromium is responsible for major environmental pollution problem in industrial and mining areas. Chromium and its derived compounds are having very high toxicological potential. Chromium listed as 17th among the most hazardous substances according to the Agency for Toxic Substances and Disease Registry (ATSDR) USA, 2017. Chromium has a variable oxidation states ranging from −2 to +6. Although chromate [Cr(VI)] can be found in the forms of (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) ions and is a widespread industrial and nuclear waste, Cr(VI) has been found to exhibit carcinogenicity, teratogenicity, and mutagenicity. Cr(VI) can easily disperse through cell membranes as it reported to has a high solubility. Chromate has a high range of toxicity since it induces the formation of reactive oxidative species and affects both DNA and protein functions after entering the cell (Ackerley et al. 2006; Balusamy et al. 2019; Shrivastava et al. 2002). Cr(VI) is also reported to make severe health issues in human, other living organisms, and ecological system as they are not easily degraded and hence come to accumulate in living cell. It is well known that Cr(VI) exposure in the environment can cause serious health problems. Acute systemic poisoning can result from high exposure to hexavalent chromium (Lin et al. 2009). Recent epidemiological study strongly points to Cr(VI) as the agent of carcinogenesis. Studies have shown that chronic exposure of Cr(VI) leads to respiratory cancer in human beings. It also shown that subcutaneous administration of chromium can cause oxidative damage, genotoxic effect, and alteration in the effects of antioxidants in experimental animals. Similarly, in the case of plants, Cr(VI) exposure can result in severe physiological problems. On the other hand, Cr(III) in its other inorganic forms is reported to be less toxic, less mobile, not easily get solubilize under neutral pH, and also cannot be able to cross cell membranes. Apart from less toxic, the trivalent chromium, Cr(III) is also found to be comparatively stable. Thus, the conversion of Cr(VI) to Cr(III) is much needed for the elimination of hexavalent form of toxic chromium from environment.

Reduction of Cr(VI) to Cr(III) can be achieved using physical, chemical, as well as biological means. Conventional removal of Cr(VI) have various drawbacks as it demands high energy cost and also generate toxic sludge that causes environmental pollution (Sen and Dastidar 2010). As compared to physico-chemical and other treatments, microbial Cr(VI) reduction is an economical and environmentally friendly process (Verma and Kuila 2019). In this aspect, chromium-reducing

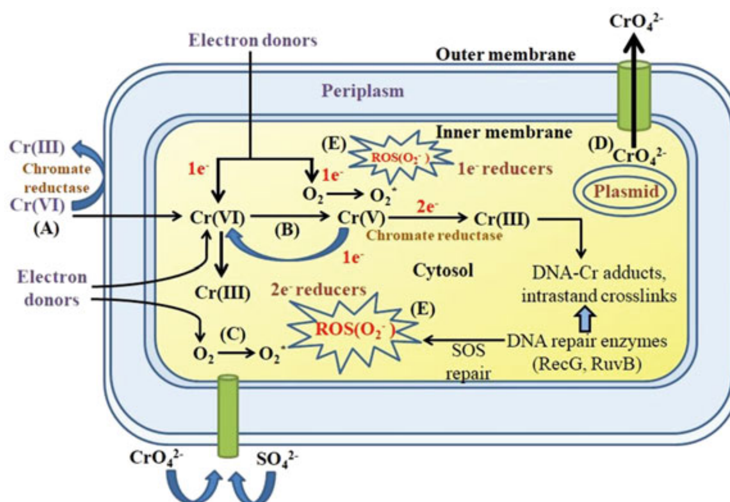

Fig. 2.1 Mechanism of enzymatic Cr(VI) reduction in the bacterial cell

bacteria (CRB) shown to act as potent biotransformer in the reduction of hexavalent chromium to non-toxic trivalent chromium. This biotansformation of Cr(VI) to Cr (III) immerged as both economical and eco-friendly. Chromium-resistant bacteria (CRB) catalyze the reduction of chromate both aerobically and anaerobically (Gao et al. 2010). There are several biotic and abiotic factors such as pH, temperature, different dose rates of chromium, incubation time, and nature of microbial species that have immense regulatory effect on Cr(VI) reduction (Wani et al. 2019; Soni et al. 2013; Narayani and Shetty 2013). Cytosolic reductases are designated as the most favorable candidate to be used in bioremediation purpose because they can be tailored easily by protein engineering (Priadie 2012; Thatoi et al. 2014). The current chapter focused on the mechanism involved in chromate reduction by bacterial chromate reductase which is considered to be one of the most efficient biomolecule utilized for the conversion of Cr(VI) to Cr(III) which has comparatively less toxicity (Fig. 2.1).

2.2 Bacteria in Chromate Reduction

Chromate reduction by bacteria was first documented by Romanenko and Koren'kov (1977) in an anaerobic bacterium *Pseudomonas dechromaticans* found in sewage sludge. *Enterobacter cloacae* obtained from industrial wastewater which is a facultative anaerobic bacterium was reported to reduce chromate (Wang et al. 1989). Aerobic Cr^{6+} reduction is generally conducted by cytosolic chromate reductase which needs the participation of an electron donor like NAD(P)H (Pradhan et al. 2016). Some of these types of cytosolic reductases have been studied by Puzon et al. (2002) in *E. coli*. Also, some other Cr^{6+} reducing aerobic bacteria have been reported

such as *P. ambigua, P. putida, Bacillus coagulans* (Ackerley et al. 2004b), *P. fluorescens* LB300 (Bopp and Ehrlich 1988), *Bacillus subtilis* (Garbisu et al. 1998), *Bacillus maroccanus* ChrA21 (Viti et al. 2003), and *Corynebacterium hoagie* ChrB20 (Viti et al. 2003; Ishibashi et al. 1990). Subsequently, A number of Cr-resistant bacteria have been found which belong to various systematic and physiological groups with increased Cr(VI)-reducing capability which include *Escherichia, Thermus, Shewanella, Bacillus*, *Enterobacter, Pseudomonas*, *Agrobacterium, Deinococcus*, *Ochrobactrum,* and many other genera (Ohtake et al. 1987; Park et al. 2000; Viti et al. 2014). Chromium-resistant bacteria are reported to reduce chromate with the help of transferring electrons from the nucleophilic cofactor such as NAD(P)H to Cr(VI). These enzymes are shown to be either cytosolic or membrane bound in the bacterial cell (Table 2.1).

2.3 Enzymatic Chromate Reduction

A variety of bacterial species have shown to retain the enzyme chromate reductase which is capable of reducing the toxic form of chromium known as chromate (Table 2.2) (Rath et al. 2014; Ackerley et al. 2004a, b; Cervantes et al. 2001; Ganguli and Tripathi 1999). NADP and FMN are the two main electron donors that are associated during the chromate reductions (Shi and Dalal 1990). Chromate-reducing enzymes were categorized into two classes, viz. LpDH and YieF as either one ($1e^-$) or two ($2e^-$) electron reducer (Ackerley et al. 2004a). ChrR of *P. Putida* and both the YieF and NfsA of *E. coli* are some chromate-reducing enzymes which reported to act as $2e^-$ reducers (Barak et al. 2006) (Table 2.2). In case of $1e^-$ reducers chromate reductase, the formation of a Cr(V) intermediate has been reported at the time of when Cr(VI) gets reduced to Cr(III). An uninterrupted conversion from Cr(VI) to Cr (V) has shown to occur with the shifting of $1e^-$ from Cr(V) to O_2. As a result of this steady conversion from Cr(VI) to Cr(V), reactive oxygen species (ROS) generation occurs in a Fenton-like reaction. Whereas in case of $2e^-$ reducers chromate reductase mediated reduction and no formation of Cr(V) has been recorded. But this type of reduction involves the transfer of $3e^-$ to Cr(VI) which result in its immediate conversion to Cr(III). Side by side $1e^-$ is also shown to be shifted to O_2 and form ROS (superoxide, O_2^{2-}).

2.4 Types of Chromate Reductases

Bacterial chromate reductases are classified according to the mode of the reduction process, i.e., aerobic or anaerobic. According to the sub-cellular localization, the enzyme chromate reductases can be found in membrane (Mala et al. 2015) or in the cytosol (Bae et al. 2005; Park et al. 2000; Suzuki et al. 1992). The reduction of hexavalent chromate is a redox reaction and requires supply of electrons which has shown to be provided by the electron donors such as NAD(P)H. Due to this reduction, a subsequent amount of reactive oxygen species (ROS) has reported to

Table 2.1 Some types of bacterial Cr(VI)-reducing enzymes and their structural characteristics

Enzyme	Type of enzyme	Class type	Organism	Mol wt. (kDa)	Structure	Reference
ChrR	Quinone reductase	I	*E. coli*		Dimer	Eswaramoorthy et al. (2012)
Gh-ChrR	Chromate reductase	I	*Gluconacetobacter hansenii*		Tetramer	Jin et al. (2012)
ChrR	Flavoenzyme	I	*P. putida*	50	Dimer	Park et al. (2000)
ChrA	Flavoprotein family of reductase	I	*P. aeruginosa, Cupriavidus metallidurans*		–	Diaz-Perez et al. (2007)
Chr	NADH-dependent	I	*R. sphaeroides*	42	Monomer	Nepple et al. (2000)
YieF	FMN or NAD(P)H dependent chromate reductase	I	*E. coli*	50	Dimer	Ackerley et al. (2004b)
OYE enzyme	Flavin oxidoreductase	I	*S. carlsbergensis*	45	Monomer	Saito et al. (1991)
Frp	Flavin reductase P	I	*Vibrio harveyi*		–	Zenno et al. (1998)
NemA	Chromate reductase	I	*E. coli*		–	Ishak et al. (2016); Robins et al. (2013)
AzoR	Azoreductase	I	*E. coli*		–	Robins et al. (2013)
Chromate reductase	NAD(P)H-dependent	I	*Bacillus* sp. RE		–	Elangovan et al. (2006)
NfsA	Nitroreductase (flavoprotein)	II	*E. coli*	50	Dimer	Ackerley et al. (2004b)
NfsB	Nitroreductase	II	*Vibrio harveyi*	50	Monomer	Kwak et al. (2003)
ChrR	NADPH dependent		*Thermus scotoductus*	72	Dimer	Opperman et al. (2008)
YcnD	FMN reductase	II	*B. subtilis*		–	Morokutti et al. (2005)
Suzuki enzyme	NAD(P)H dependent chromate reductase	II	*P. ambigua*	65	Dimer	Suzuki et al. (1992)
EcdA	Soluble chromate reductase	II	*P. putida*		–	Park et al. (2000)
YdgL	Nitroreductase		*Bacillus subtilis*		–	Minton et al. (2004) (patent)
KefF	Nitroreductase	II	*E. coli*		–	Prosser et al. (2010)
Chr BAC	Efflux transporter		*Shewanella* strain ANA-3		–	Aguilar-Barajas et al. (2008)

(continued)

Table 2.1 (continued)

Enzyme	Type of enzyme	Class type	Organism	Mol wt. (kDa)	Structure	Reference
25 kDa protein	Cell free extract (inducible)		*Bacillus* sp. JDM-2-1		–	Zahoor and Rehman (2009)
FRase 1	FMN reductase	II	*Vibrio* (or *Photobacterium*) *fischeri*		–	Zenno et al. (1994)
ChrT	NADPH dependent FMN reductase		*Serratia* sp.		–	Zhou et al. (2017)

Table 2.2 Involvement of chromate reductases in one- and two-electron reduction mechanism

Reduction types	Mechanism employed for chromate reduction	Examples
One-electron reducers	Reduction of Cr(VI) to Cr(III) occurs via Cr (V) intermediate. A continuous shuttle between Cr (VI) and Cr(V) forms happens with Cr(V) transferring $1e^-$ to O_2, generating ROS (superoxide, O_2^{2-}) in a Fenton-like reaction (Shi and Dalal 1990; Barak et al. 2006)	LpDH
		Cytochrome c
		Glutathione reductase
		Ferredoxin-NADP oxidoreductase
		NfsA (*E. coli*)
Two-electron reducers	Transfer of $3e^-$ to Cr(VI) results in its direct reduction to Cr(III) $1e^-$ is transferred to O_2, forming ROS (superoxide, O_2^{2-}). No Cr(V) intermediate is involved hence no redox cycle occurs	ChrR (*P. putida*)
		YieF (*E. coli*)

be formed. Two different types of reaction mechanism catalyzed by chromate reductase have been proposed which are Class I "tight" and Class II "semi-tight" according to the pathway of electronic transmission to Cr(VI) and the quantity of ROS produced. All Class I chromate reductase enzymes are effective chromate and quinone reducers, while the Class II chromate reductases are reported to potentially mediate the reduction of quinones and nitro compounds (Park et al. 2002).

2.4.1 Nitroreductase

The enzyme nitroreductase (Nfs) isolated from the bacterium *Shewanella oneidensis* MR-1 has shown to possess both the aerobic and anaerobic chromate reduction activities (Viamajala et al. 2002). The chromate reduction process catalyzed by this enzyme is reported to produce more ROS than reduction by ChrR (Ackerley et al. 2004b). The reaction mechanism of this enzyme suggested that nitroreductase reduces chromate with the help of the cofactor NAD(P)H alike ChrR (Kwak et al. 2003).

2.4.2 Quinone Reductase

The crystal structure of ChrR enzyme of *Escherichia coli* was solved at 2.2 Å resolution (Eswaramoorthy et al. 2012). Analysis of this crystal structure revealed that the enzyme is a tetramer, and in its tetrameric form, the enzyme catalyzes chromate reduction. Within this tetrameric structure, there are some residues shown to be responsible for making a pair of two hydrogen bond networks, formed by Tyr128 and Glu146 in one dimer and Arg125 and Tyr85 present in other dimer (Ackerley et al. 2004a, b; Gonzalez et al. 2005).

Structurally, ChrR of *E. coli* belongs to the FMN reductase family of flavodoxin superfamily because this enzyme shares the flavodoxin-like fold (Carey et al. 2007; Deller et al. 2006; Ackerley et al. 2006; Gonzalez et al. 2005). EcChrR shown to have the closest structural similarity with the putative FMN reductase of *P. aeruginosa* PA01 T1501 (PDB ID: 1RTT) which has a tetrameric structure. The hydrogen bond pattern at the dimer-dimer coherence site present in the tetrameric form of *P. aeruginosa* FMN reductase also resembles with *E. coli* ChrR (Agarwal et al. 2006).

2.4.3 Iron Reductase

Schmidt (1996) in his study showed the chromate reduction activity of iron reductase [Fe(III) reductase] in a flowering plant species, *Plantago lanceolata*. Another bacterial iron reductase enzyme known as FerB from *Pseudomonas denitrificans* has shown to have chromate-reducing ability. This enzyme reported to use Fe(III) nitrilotriacetate and chromate as substrates during its catalysis of chromate reduction (Mazoch et al. 2004).

2.4.4 Flavin Reductase

Puzon et al. (2002) in their study reported about a bacterial flavin reductase that is able to reduce chromate in a soluble chromium(III)-NAD^+ conjugate which is an end-product of this reduction process. The enzyme flavin reductase has shown to bind with a biomolecule called flavin mononucleotide (FMN) during the reduction of chromate (Gonzalez et al. 2003).

2.4.5 NAD(P)H-Dependent FMN Reductase

Suzuki et al. (1992) in their study has reported about the NADP(H)-dependent FMN reductase in chromium reduction in the bacterial species *Pseudomonas ambigua* G-1. This enzyme is classified under the family of flavoprotein. Kinetic analysis of the enzyme reported a wide range of temperatures (40–70 °C) and pH (6–9) for the optimum enzyme activity. Agarwal et al. (2006) solved the X-ray crystallographic structure of NAD(P)H-dependent FMN reductase from the bacterial species *Pseudomonas aeruginosa* PA01 at 1.28 Å resolution. Detailed analysis of the solved crystal structure of this protein revealed that this enzyme has an FMN-binding site "GSLRSGSYN." Structural comparison between the FMN unbound and FMN bound conformer of the enzyme has also been performed in this work which implied that the enzyme shows conformational changes on cofactor (NADPH) binding. Agarwal et al. (2006) confirmed the NADPH-dependent activity by performing the biochemical assays.

2.4.6 Aldehyde Oxidase

Banks and Cooke Jr (1986) reported the chromate reduction by rabbit liver aldehyde oxidase. Aldehyde oxidase is enzyme under flavoenzyme family which contains molybdenum and can be found in different organs as well as tissues of many mammals, including humans. This enzyme is also shown to have the capability to oxidize or reduce different xenobiotic compounds (Jakoby 2012).

2.4.7 C-Type Cytochrome

A number of sulfate-reducing bacteria (SRB) have been reported to be responsible for the reduction of chromate by their periplasmic c-type cytochrome (Elias et al. 2004; Xia et al. 2018). *Desulfovibrio vulgaris* Cytochrome c3 (Lovley and Phillips 1994), *Desulfuromonas acetoxidans* cytochrome c7 (Michel et al. 2001), *Acidiphilium cryptum* JF-5 c-type cytochrome ApcA shown to have chromate-reducing efficiency (Magnuson et al. 2010). There are two extracellular c-type cytochrome, namely MtrC and OmcA found in the bacterial species *Shewanella oneidensis* MR-1 (Reardon et al. 2010) are shown to have an elevated capacity to reduce chromate (Belchik et al. 2011). Because of the homologous tetrahedral structure of sulfate and chromate, these two compounds (sulfate and chromate) share the similar binding site, and thus chromate can interact with the cytochrome c of SRB (Assfalg et al. 2002). Cytochrome c3 reported to possess four hemes, and they are labeled as I, II, III, and IV (Higuchi et al. 1984). The heme II is absent in cytochrome c3 which has shown to be present in cytochrome c7 (Czjzek et al. 2001). ApcA is only cytochrome c reported to contain a single heme (Magnuson et al. 2010), and it is also shown to reduce chromate with step-by-step oxidation reaction. MtrC and OmcA are shown to be differing from other two cytochromes and shown to contain 10 hemes (Shi et al. 2006).

2.4.8 DT-Diaphorase

De Flora et al. (1985) in their study investigated the role of DT-diaphorase in reducing Cr(VI) in rat liver. They have found that the enzyme activity is NADPH dependent. Aiyar et al. (1992) have also shown that rat liver cytosolic and microsomal fractions lead to the reduction of Cr(VI) to Cr(V). The result from their study represents that the enzymatic action of DT-diaphorase indirectly decreases Cr(VI)-mediated mutagenicity in *Salmonella* TA100 tester strain which was used as a model for Ames test. The reason behind this enzymatic reduction of Cr(VI) to Cr(V) is possibly through the interaction of DT-diaphorase enzyme with other redox active cellular elements.

2.4.9 Hydrogenase

Chardin et al. (2003) reported the efficiency of sulfate reducing bacteria (SRB) to reduce Cr(VI). In this study, they demonstrated that the hydrogenases obtained from SRB like *Desulfovibrio* and *Desulfomicrobium* have highest Cr(VI)-reducing ability. Moreover, the [Fe] hydrogenase from the strain Hildenborough of *Desulfovibrio vulgaris* reported to have highest Cr(VI)-reducing rate. In an experiment conducted using electron paramagnetic resonance spectroscopy, Chardin et al. showed that Cr (VI) gradually become reduced to paramagnetic Cr(III) by the enzyme [Fe] hydrogenase. The reduction rate by [Fe] hydrogenase shown to be decreased with the increase in Cr(VI) concentration (Fig. 2.2).

2.5 Structural and Functional Analysis of Different Reductases

A number of investigations performed on enzymes that take part in the reduction of chromate. Patra et al. (2010), in their investigations, performed the molecular characterization of chromium (VI) reducing gram positive bacteria isolated from contaminated sites. Information about amino acid sequence of protein, function, sub-cellular localization, active site, mutagenesis study of these enzymes is found in UniProt protein database (http://www.uniprot.org). Although, a limited number of investigations have been performed for understanding the structural and functional characteristics of these reductases (Table 2.3). The crystal structure of several chromate reductase-related enzymes from the bacterial species *Thermus scotoductus*, *Escherichia coli*, and *Gluconacetobacter hansenii* and nitroreductase from *Desulfovibrio alaskensis*, *Bacillus subtilis, Escherichia coli,* and *Paracoccus denitrificans* has been solved. The 3D structure of the enzyme nitroreductase from *E. coli* has been solved by Lovering et al. (2001). The analysis of the structural characteristics of this enzyme showed that the enzyme is dimeric and has a large hydrophobic core. Each monomer of the enzyme consists of five β-sheets surrounded by α-helices. Catalytic mechanism of the enzyme is shown to be induced by nitrite compounds. Eswaramoorthy et al. (2012) determined the 3D structure of quinone reductase form *Escherichia coli* strain K12 which was reported to belong from the flavodoxin superfamily. Analysis of this crystal structural revealed that the flavin mononucleotide (FMN) remains firmly attached to the active site of the enzyme and plays a role of the substrate molecule. During the functioning of this enzyme, transfer of electron occurs from NADH to different other substrates along with FMN by a mechanism called “ping-pong bi-bi reaction.” Structural analysis of the enzyme iron reductase from *E. coli* strain 042 showed that the cofactor of this enzyme flavin adenosine-dinucleotide (FAD) remains juxtaposed with the substrate NAD(P)H which causes nucleophilic attack to the substrate during the enzyme-mediated reduction (Tomasiak et al. 2011). Rangarajan et al. (2004) solved the crystal structure of enzyme flavin reductase from the bacterial strain *E. coli* O157: H7, which is shown to contain a typical Rossmann fold in which FMN remains attached. Study of the catalytic mechanism of this enzyme reported that NADH and

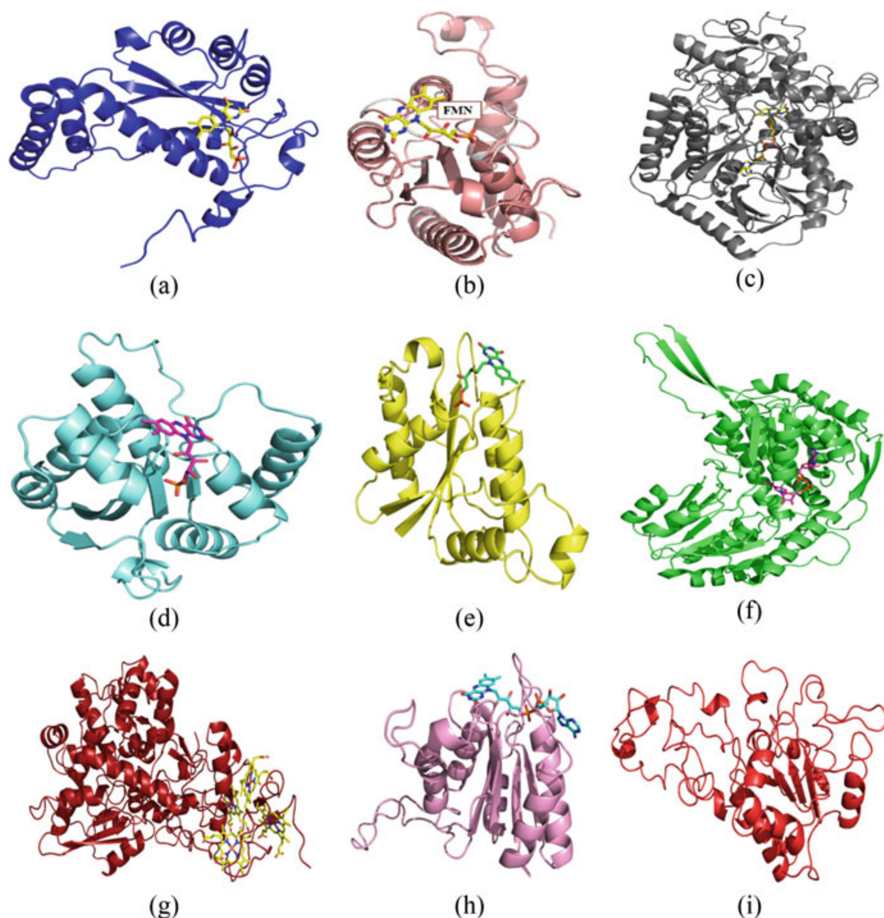

Fig. 2.2 3D structures of different chromate reductase–related enzymes. (**a**) Nitroreductase of *E. coli* (PDB ID: 1ICV); (**b**) Quinone reductase of *E. coli* (PDB ID: 3SVL); (**c**) Iron reductase of *E. coli* (PDB ID: 3P4R); (**d**) Flavin reductase of *E. coli* (PDB ID: 1SBZ); (**e**) NAD(P)H-dependent FMN reductase of *Pseudomonas ambigua* (PDB ID: 1X77); (**f**) Aldehyde oxidase of *E. coli* (PDB ID: 1WNB); (**g**) C-type cytochrome of *Desulfovibrio vulgaris* (PDB ID: 1GX7); (**h**) DT-diaphorase of *E. coli* (PDB ID: 2B3D); (**i**) Hydrogenase of *Desulfovibrio vulgaris* (PDB ID: 5JSH)

glutathione take part in the reduction process. The solved crystal structure of the enzyme aldehyde oxidase from *E. coli* strain K12 reveals that it is a tetramer, and each monomer binds with NADH (Gruez et al. 2004). Elantak et al. (2003) discovered the three-dimensional structure of c-type cytochrome from the bacterium *D. vulgaris*. Studies about the enzyme structure have shown that the interacting surface of c3 cytochrome and this enzyme is close to the position of heme moiety present in c3 cytochrome. The structure of the enzyme DT-diaphorase from *E. coli* has been solved by Adams and Jia (2006), where they reported that the enzyme contains a structural fold consists of a twisted β-sheet positioned centrally which is

Table 2.3 Types of chromate reductase family enzymes, their catalytic mechanism, and structural characteristics

Enzyme name	Catalytic mechanism	PDB ID	Structural characteristics	References
Nitroreductase	Induced by nitrite	1ICV	The enzyme is dimeric and has a large hydrophobic core; each half of the molecule consists of a 5 β-sheets surrounded by α-helices	Lovering et al. (2001)
Quinone reductase	Electron transfer occurs from NADH to the various substrates via ping-pong bi-bi reaction	3SVL	It belongs to the flavodoxin superfamily in which flavin mononucleotide (FMN) is firmly anchored to the protein	Eswaramoorthy et al. (2012), Gonzalez et al. (2005), Carey et al. (2007), Deller et al. (2006)
Iron reductase	Reduction is mediated in the presence of NAD (P)H	3P4R	Structural analysis shows that the cofactor FAD remains very close to the substrate NAD(P)H which causes nucleophilic attack to substrate	Schröder et al. (2003), Tomasiak et al. (2011)
Flavin reductase	Reduction is catalyzed by NADH and glutathione	1SBZ	The enzyme consists of a typical Rossmann fold containing a non-covalently bound molecule of FMN	Puzon et al. (2002), Rangarajan et al. (2004)
NAD(P)H-dependent FMN reductase	During the reduction, 1 mol of Cr(VI) converts to Cr(III) in expense of 3 mol of NADH as an electron donor	1X77	Contains a novel flavin mononucleotide-binding site "GSLRSGSYN." The enzyme show conformational changes on cofactor (NADPH) binding	Suzuki et al. (1992), Agarwal et al. (2006)
Aldehyde oxidase	This enzyme uses aldehydes or heterocyclic imines as electron donor substrates such as NADH	1WNB	The solved crystal structure of this enzyme revealed that it is a tetramer and each monomer binds with NADH molecule	Banks and Cooke Jr (1986), Jakoby (2012), Gruez et al. (2004)

(continued)

Table 2.3 (continued)

Enzyme name	Catalytic mechanism	PDB ID	Structural characteristics	References
C-type cytochrome	Induced by chromate	1GX7	The interacting surface between cytochrome c3 and the enzyme is close to the heme4 of cytochrome c3 and is surface exposed	Magnuson et al. (2010), Elantak et al. (2003)
DT-diaphorase	Enzyme activity is NADPH dependent	2B3D	The enzyme has a structural fold consists of a twisted central parallel β-sheet that is surrounded by α-helices	De Flora et al. (1985), Adams and Jia (2006)
Hydrogenase	Cr(VI) reduction rates by this enzyme are directly correlated with hydrogen consumption rates	5JSH	According to experimental data and molecular dynamics simulations of hydrogenases, the most probable proton transfer pathway involves a glutamate residue (Glu28 in the *D. vulgaris*) that receives protons from the active site	Baltazar et al. (2012), Chardin et al. (2003), Dementin et al. (2004), Marques et al. (2017)

surrounded by α-helices. Marques et al. (2017) reported the crystal structure of the enzyme hydrogenase from *D. vulgaris* as well as the dynamics of that enzyme using molecular dynamics simulations techniques. They hypothesized from the solved crystal structure and the dynamism study of the enzyme that the proton transfer pathway during the enzyme-mediated catalysis involved the participation of a glutamate residue (Glu28 in the *D. vulgaris*) which accept the proton from the active site of hydrogenase enzyme.

The crystal structure of ChrR from a thermophilic bacterium *Thermus scotoductus* has been solved. The three-dimensional structure represents a typical $(\alpha/\beta)_8$ TIM-barrel structure consists of eight twisted β-strands bordered by eight α-helices. This signature TIM-barrel structure is found in all the homolog enzymes classified under the family of Old Yellow Enzyme (OYE). The most similar structural relative of ChrR of *T. scotoductus* is XenA and YqjM with an RMSD of 0.9 Å and 1.1 Å, respectively. The structure of *T. scotoductus* ChrR represents a number of characteristics, loop and a β-hairpin. The largest loop in the enzyme structure is formed between β3 and α3. The analysis of the *T. scotoductus* ChrR structure also

revealed the presence of a region known as "capping domain" which contains a parallel and an antiparallel β-sheet (Opperman et al. 2008; Opperman and Heerden 2008). This region of *T. scotoductus* ChrR has very less conservation among the related homologs structures of other enzymes. Loop region of *T. scotoductus* ChrR is shown to retain a greater number of proline residues compared to its mesophilic counterparts YqjM and XenA (Williams et al. 2004). This comparatively greater number of proline content in the loop region of *T. scotoductus* ChrR is reported to decrease the configurational entropy which ultimately gives the structural stabilization in *T. scotoductus* ChrR even at a high temperature relative to the homolog enzymes of mesophilic counterparts.

The above-mentioned all the structural and functional aspects of chromate reductase family enzymes could promote the strengthening of concept about the enzyme and its intrinsic mechanism of action. A vigorous knowledge about the structure of enzymes and the interrelationship of its structure–function can make an enzyme easily mutated through protein engineering approach for promoting its catalytic efficiency. Therefore, it is very apparent that the structural as well as functional variability of different microbial chromate reductase requires in-depth investigations for a proper understanding about enzyme-mediated catalysis in the view of effective bioremediation of chromium.

2.6 Conclusions

The enzymes that are found effective in the reduction of Cr(VI) have been investigated in a number of microorganisms and are classified under different family such as quinone reductases, NADPH-dependent FMN reductase, nitroreductases, iron reductase, hydrogenase, etc. Several bacteria have shown to reduce Cr(VI) with the help of membrane attached enzymes like flavin reductase, cytochromes, and hydrogenase which involved in electron transport chain and utilize chromate as the terminal electron acceptor. Bacterial strains belong to different genera also are shown to retain cytosolic chromate reductase activity. NAD(P)H has shown to play the role of electron donor during the chromate reductase–mediated reduction of Cr(VI). In comparison with membrane-bound chromate reductases, cytosolic reductases are reported to be favorable for their use in bioremediation. In addition, these cytosolic chromate reductase enzymes were shown to be easily modified by protein engineering to make them adapt environmental conditions of contaminated sites. Although many of the bacterial cytosolic chromate reductases have been reported, the purification and structural–functional characterization have been performed for only a few of them. In a small number of cases, genes that take part in the production of chromate reductase have been recognized. Therefore, a more and vast investigations and understanding are still needed for the exploitation of in-depth enzymatic reaction related to chromate reduction and their optimized application in toxic chromium bioremediation in environment.

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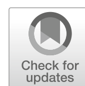

3 An Overview of Raw Starch Digesting Enzymes and Their Applications in Biofuel Development

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Abstract

With an ever-increasing global population, the demand for energy is growing at a faster pace. Conversely, fossils fuels are getting depleted much faster, and it has been estimated that, together with all the fossil fuel reserves, they cannot sustain the escalating demand for energy even until 2100 AD. The current impetus is therefore directed toward the search for alternative sources of sustainable energy, i.e., bioenergy. Bioethanol, biobutanol, and biohydrogen constitute a major fraction of bioenergy and are considered as clean energy owing to their nearly complete combustion without the release of toxic gases to the environment. One of the raw materials used for these biofuel productions is starch; however, conventional starch conversion into biofuel is an energy-intensive process, adding more cost to the final product. An alternative approach is the cold hydrolysis of raw starch by granular/raw starch digesting enzymes (RSDE) which require less energy as it bypasses the starch liquefaction steps. RSDE are a group of amylases that can act on native granular starch at mesophilic

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conditions. Although RSDE could be produced from different sources, microbial production is the most economical and advantageous. Thus, the present review aims to bring together the recent progress in RSDE research focusing on microbial production, purification, characterization, protein engineering, and applications.

Keywords

Starch · Amylolytic enzymes · Extremophiles · Hydrolysis · Biofuels

3.1 Introduction

Demand for energy is increasing rapidly with the explosion in global population. The consumption of energy has reached up to 13-fold in the twentieth century, much greater than the population growth (Gupta and Verma 2015). Over 11 billion tons of oil is estimated to be consumed annually, and if the demand persists, there will be hardly any oil deposits left by 2052 (Ecotricity Group Limited 2018). Even other fossil fuel reserves such as coal and natural gas might not sustain beyond the year 2088 (Ecotricity Group Limited 2018). The current impetus is therefore channeled toward the search for alternative sources of sustainable energy, i.e., bioenergy.

Bioethanol is one of the major biofuels currently in use, and its production is expected to reach 134.5 billion liters by 2024 (Wissner et al. 2015). The use of bioethanol in various sectors is also increasing, principally due to the environmental concerns raised over the toxic gas emissions from petroleum fuels. A 5% ethanol blend to gasoline can reduce CO_2 and SO_2 emissions by 90% and 60–80%, respectively (Saxena et al. 2009). This corresponds to approximately 41.2 million metric tons of CO_2-equivalent greenhouse gas emissions from transportation which is equivalent to 8.7 million cars off the road for an entire year (Renewable fuels Association 2016). Another important biofuel is biobutanol, which is much cleaner than bioethanol. With high energy content, hydrophobicity, blending ability, compatibility to combustion engines, octane rating, and being less corrosive, biobutanol offers a promising alternative as biofuel (Kumar and Gayen 2011). Since its recognition as a novel biofuel in 2005, biobutanol production has gained momentum (Kumar and Gayen 2011) and is anticipated to cover the global demand for gasoline and diesel by approximately 20% (Nanda et al. 2017). Among other biofuels, biohydrogen is the most advancing energy carrier owing to its clean and low emission, particularly greenhouse gases (Boodhun et al. 2017). Hydrogen, being a cleaner, colorless, odorless, and tasteless fuel with only water as the dissociation product, forms a sustainable, pollution-free substitute for the shrinking fossil fuels (Boodhun et al. 2017). It is anticipated that biohydrogen will contribute a 10% share to the total energy market by 2025 (Boodhun et al. 2017).

Currently, USA and Brazil are the leading producers of biofuel, especially bioethanol, and constitute approximately 56.72% and 26.72%, respectively, of the world's total ethanol production (Gupta and Verma 2015). Bioethanol is mainly

produced by the fermentation of sugars extracted from sugarcane in Brazil or corn starch in the USA (Shanavas et al. 2011). The conventional process for biofuel production from starchy material involves four steps, viz. gelatinization (80–125 °C), liquefaction (80–110 °C), saccharification (60–70 °C), and fermentation (30–32 °C). The first three steps convert starch into fermentable sugars by α-amylase and glucoamylase, while the last step is usually performed by microbes such as yeast/*Saccharomyces*/*Clostridium* sp. (Vendruscolo 2015; Moshi et al. 2015). However, the first three steps are energy-intensive (Mehta and Satyanarayana 2013) which escalate production costs (Sun et al. 2009) and warrants the need for the reduction of energy consumption. Further, if starch hydrolysis is undertaken below the gelatinization temperature, then the viscosity problem can be minimized (Goyal et al. 2005). This has led to the search for raw starch digesting enzymes (RSDE) which can hydrolyze raw starch granules directly below their gelatinization temperature (Goyal et al. 2005; Sun et al. 2010). The decrease in temperature during starch digestion by the use of RSDE can save up to 10–20% of the total energy in biofuel production (Sun et al. 2010). However, a single enzyme cannot hydrolyze raw starch granules completely; a synergy of other enzymes is essential (Sun et al. 2010). Raw starch digesting α-amylase (RSDA) is an endo α-1,4 glucanase that randomly hydrolyzes the α-1,4 glycosidic linkages and destroys the starch structure rapidly (Sun et al. 2010). Previously, most of the RSDA has shown a strong correlation between the raw starch hydrolysis and enzyme adsorption; however, this is no longer considered an obligatory requirement (Mitsuiki et al. 2005). Since RSDA cannot completely hydrolyze starch, the addition of glucoamylase is essential to complete the task.

The RSDE-producing microorganisms are widely distributed in nature, among them fungi, yeasts, and bacteria are the most predominant (Sun et al. 2010). The microbes that proliferate on rotting starchy material are the most common RSDE producers; however, they have also been isolated from soil, air, mud, compost, hot springs, etc. (Sun et al. 2010). The potent producers of RSDE include yeast such as *Cryptococcus* sp., fungi such as *Aspergillus* sp. and *Rhizopus* sp., bacteria from the genus *Alicyclobacillus* sp., *Bacillus* sp., and *Geobacillus* sp., and few archaea such as *Halorubrum* sp. (Bai et al. 2012; Moshfegh et al. 2013). In the last few decades, numerous RSDE have been purified and characterized that are suitable for various industrial applications. But recently, RSDE has gained a great deal of attention for biofuel development owing to environmental concerns and the global fossil fuel crisis (Bai et al. 2012). Therefore, looking at the large prospects of RSDE, this chapter focuses on the recent progress in the understanding of microbial RSDE in the last lustrum.

3.2 Starch (Amylum): The Energy Storehouse

Starch is the chief energy source for non-photosynthetic organisms and for plants, it is the main food reserve (Hamilton et al. 2000) and forms a central component of the human diet. Apart from this, starch can be processed both chemically and

enzymatically into a variety of products for their various uses in starch processing, food-feed, textile, detergent, and paper-pulp industries (Zyl et al. 2012). Furthermore, the glucose monomer from starch can be fermented to ethanol. Though many plants produce starch, only a few plant sources are deemed important for starch processing industries. The principal starch sources are wheat, maize, cassava, and potato; however, there are limitations for their use in certain food applications. The potential reasons include low shear resistance, thermal decomposition, thermal resistance, and high tendency toward retrogradation (Goyal et al. 2005). Starch is primarily composed of two kinds of glucose polymers, viz. amylose and amylopectin. In amylose, glucose units are connected by α-1,4-glycosidic linkage as a linear polymer, while amylopectin is made up of a linear chain of α-1,4-glycosidic linkage and side chains of α-1,6 linkages (van der Maarel et al. 2002).

3.3 Starch Digesting Enzymes

Several microbes produce extracellular or intracellular enzymes to digest starch or glycogen to glucose. The starch digesting amylolytic enzymes are glycoside hydrolases (GHs), which mainly act on α-(1,4) and/or α-(1,6) linkages of starch polymers. Most of the α-amylases are grouped under GH family 13 (EC 3.2.1.1) (MacGregor et al. 2001), while β-amylases (EC 3.2.1.2) and glucoamylases (EC 3.2.1.3) are grouped into GH14 and GH15 families, respectively (Christiansen et al. 2009). With respect to the mode of action, the starch-converting enzymes are grouped into mainly four groups: (1) endoacting, (2) exoacting, (3) debranching, and (4) transferases. Endoacting enzymes are α-amylases that randomly cleave α-1,4-glycosidic bonds in amylose, amylopectin, and related polysaccharides and produce oligosaccharides of varying chain lengths with α-configuration on C1 of the newly formed reducing end glucose unit (Liu et al. 2010) while exoacting amylolytic enzymes such as β-amylases (EC 3.2.1.2), glucoamylases (EC 3.2.1.3), or α-glucosidases (EC 3.2.1.20) act specifically on α-1,4 glycosidic linkages of starch from the non-reducing end and produce glucose or low molecular weight oligosaccharides (Sivaramakrishnan et al. 2006). The third group of enzymes that hydrolyze starch is the debranching types such as isoamylase (EC 3.2.1.68) and pullulanase type I (EC 3.2.1.41) that exclusively hydrolyze α-1,6 glycosidic linkages (van der Maarel et al. 2002). The major difference between these debranching enzymes is their preference for pullulan (Israilides et al. 1999). Transferases are the fourth group which breaks the α-1,4-glycosidic bond of the donor molecule and creates a new glycosidic bond by transferring a part of the donor molecule to a glycosidic acceptor (van der Maarel et al. 2002). Examples of enzymes that form new α,1–4 glycosidic bond include cyclodextrin glycosyltransferase (EC 2.4.1.19) and amylomaltase (EC 2.4.1.25), while branching enzyme (EC 2.4.1.18) forms a new α-1,6-glycosidic bond. Enzymes falling into these groups, along with their modes of action on the starch polymer, are shown in Fig. 3.1.

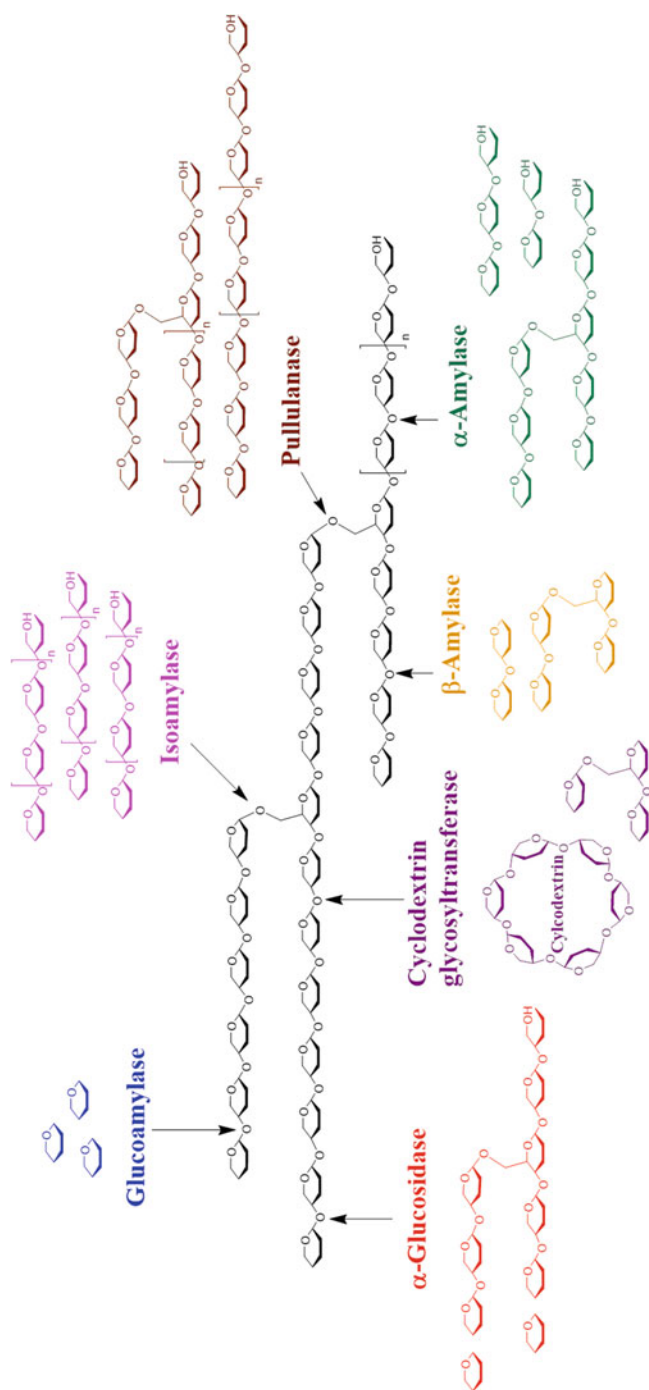

Fig. 3.1 Schematic representation of the site of action on amylopectin molecules by various amylolytic enzymes. Enzyme involved in amylopectin hydrolysis and their hydrolytic products are marked with the same color

3.4 Raw Starch Digesting Enzymes

Raw starch digesting enzymes (RSDE) or granular starch hydrolyzing enzymes refer to amylolytic enzymes that may fall under any of the above four categories which directly breakdown raw starch granules under their gelatinization temperature (Nwagu et al. 2012). RSDE have been reported to be a type of α-amylase, amylopullulanase, cyclomaltodextrin glucanotransferase, β-amylase, and glucoamylase which act on raw starches (Sun et al. 2010). Among the different amylolytic enzymes, only 10% are able to breakdown linkages in raw or granular starch (Zyl et al. 2012) with most of them containing a specific carbohydrate-binding site, the starch binding domain (SBD) (Coutinho and Reilly 1997). It is perceived that SBD brings the enzyme to the proximity of the substrate, which would increase the efficiency of catalysis (Cornett et al. 2003). However, it was established that SBD does not seem to be a mandatory requirement for RSDE action (Puspasari et al. 2013; Roy et al. 2013). Some enzymes on their surface contain the surface binding sites, built of aromatic amino acids like tryptophan and tyrosine, which provide the enzyme–starch association via stacking interactions (Machovic and Janecek 2006). Although raw starch degrading organisms employ a single amylolytic enzyme for hydrolysis, a combination of various RSDE would prove to be beneficial (Zyl et al. 2012). α-Amylases can act on the surfaces of starch granules and supply glucoamylases with shorter dextrins to produce glucose as the end-product. Further hydrolysis may result in the formation of small holes in granular starch, allowing α-amylase to enter the interior of the starch molecule (Zyl et al. 2012). Thus, the raw starch digesting α-amylases and glucoamylases together may completely hydrolyze raw starch into glucose units (Zyl et al. 2012).

3.5 Sources of RSDE

Raw starch digesting amylases are ubiquitous and can be found in the animal, plant, and microbial kingdom (Sun et al. 2009). However, microbial RSDE is more desired due to cost-effectiveness, consistency, scalability, less time and space requirement, easiness in optimization, and process modification (Sun et al. 2009). Fungi, yeasts, and bacteria that produce RSDE were mainly isolated from starchy material and soil (Table 3.1); however, microbes have also been isolated from various extremophilic niches such as saltern (Mesbah and Wiegel 2014), volcanic environment (Finore et al. 2011), deep-sea biosphere (Jiang et al. 2015) and hot springs (Bekler and Güven 2014). What makes such organisms produce enzymes at extremophilic conditions is still unclear. RSDE production, like other enzymes, is also affected by strain, medium composition, and growth conditions. A review in this regard was published (Sun et al. 2009, 2010); however, in the present chapter, we attempt to analyze the recent advancements in RSDE production.

Table 3.1 Raw starch digesting enzyme producers and their growth conditions

Organism	Isolation source	Fermentation conditions		Enzyme production	References
		Carbon/nitrogen	pH/temperature (°C)		
Fungi					
Aspergillus spp.	Spoilage of cassava	Soluble starch/NH_4SO_4, $NaNO_2$	6.5/30.0	–	Avwioroko (2015)
Aspergillus sp. MZA-3	Wild cassava Rhizosphere	Potato starch/tryptone	5.5/30.0	5.5 U/mL	Moshi et al. (2015)
A. carbonarius	Rotten cassava tubers	Corn starch/yeast extract	–/30.0	–	Nwagu et al. (2012)
A. flavus	Decomposed cassava peels	Cassava starch/NH_4NO_3	6.5/37.0	2.5 U/mg	Adeyanju et al. (2014)
A. niger HQU-3	Mildewed sweet potato roots	Sweet potato starch/NH_4SO_4	4.6/28.0	121.6 U/g	Zhang et al. (2013)
A. niger F-01	Spoilage of cassava	Cassava starch/peptone, yeast extract, maltose, stearic acid ester (inducer)	7.0/34	192 U/mL	Sun and Peng (2017)
Penicillium sp. GXU20	Soil	Wheat bran/soybean meal	5.0/28	20 U/mL	Lin et al. (2011)
Rhizopus oryzae WCS-1	*Nuruk* (Korean food)	Soluble starch/yeast extract, peptone	–/30.0	–	Jang et al. (2015)
R. microsporus TISTR 3531	Thai traditional inoculum for alcoholic fermentation	Rice bran, corncob, cassava bagasse	6.0/35	389.5 U/g	Trakarnpaiboon et al. (2017)
Bacteria					
Alicyclobacillus sp. A4	Hot spring	Soluble starch/yeast extract, peptone	3.0/60.0	2.3 U/ml	Bai et al. (2012)
Amphibacillus sp. NM-Ra2	Hypersaline lake mud	Soluble starch/yeast extract	7.5–8.0/48.0	–	Mesbah and Wiegel (2014)
Anoxybacillus sp. *KP1*	Hot spring	Soluble starch/beef extract, casamino acid	8.0/50.0	14310.6 U/mL	Bekler and Güven (2014)
Bacillus amyloliquefaciens B5	Solid-state fermented media for vinegar production	Soluble starch/tryptone	4.5/–	–	Liu et al. (2015)

(continued)

Table 3.1 (continued)

Organism	Isolation source	Fermentation conditions		Enzyme production	References
		Carbon/nitrogen	pH/temperature (°C)		
B. aquimaris MKSC 6.2	Soft coral	Rice starch/peptone, yeast extract	7.0/30.0	–	Puspasari et al. (2011)
B. licheniformis ATCC 9945a	–	Soluble starch/tryptone	6.5/37.0	5.2 U/mL	Božić et al. (2011)
B. licheniformis AS08E	Soil	Soluble starch/peptone	11.5/45.0	162.9 U/mL	Roy and Mukherjee (2013)
B. megaterium VUMB109	–	Soluble starch/$(NH_4)_2HPO_4$	8.2/40.0	20 U/mL	Jana et al. (2013)
B. mojavensis A21	Marine water	Chicken feathers, yeast extract	7.0/37.0	4.78 U/mL	Hmidet et al. (2010)
B. subtilis AS01a	Soil	Soluble starch/beef extract	6.1/45.0	799 U/mL	Roy et al. (2012)
B. subtilis S8–18	Marine sediments	Soluble starch/tryptone, yeast extract	7.0/37.0	–	Kalpana and Pandian (2014)
B. subtilis JS-16	Sodic sludge	Soluble starch/yeast extract	9.0/37.0	1.08 U/mL	Menon et al. (2014)
B. subtilis BI19	Soil	Rice starch/peptone	8.0/37.0	8.67 U/mL	Dash et al. (2015)
Geobacillus sp. IIPTN	Hot spring water	Soluble starch/soybean meal, yeast extract	7.0/60	192 U/mL	Dheeran et al. (2010)
Geobacillus sp. 4j	Deep-seafloor sediments	Soluble starch/yeast extract	6.8/60	–	Jiang et al. (2015)
G. thermoleovorans NP33	Hot spring water	Soluble starch/yeast extract	7.0/70	16.4 U/mL	Nisha and Satyanarayana (2014)
G. thermoleovorans subsp. *stromboliensis* subsp. nov.	Geothermal volcanic environment	Soluble starch/yeast extract	5.6/70	20,000 U/mL (I_2)	Finore et al. (2011)

Halolactibacillus sp. SK71	Saline soil	Soluble starch/peptone, yeast extract	8.0/35.0	123.4 U/mL	Yu and Li (2014)
Laceyella sacchari LP175	Soil	Cassava starch/soya bean meal	7.0/50	86.1 U/mL	Lomthong et al. (2015)
Lactobacillus fermentum 04BBA19	Flour mixed soil	Corn flour/soya bean meal	6.0/45	732.3 U/mL	Fossi et al. (2011)
Microbacterium aurum B8.A	Sludge (potato starch-processing)	Potato starch/tryptone	7.2/37.0	–	Sarian et al. (2012)
Archaea					
Halorubrum xinjiangense	Hypersaline lake	Wheat starch/peptone	7.0–8.0/40	0.7 U/mL	Moshfegh et al. (2013)

3.6 RSDE Production Parameters

RSDE production by microorganisms can be influenced by various parameters such as carbon–nitrogen ratio, pH, temperature, etc. Soluble starch and various raw starches are the most preferred choices for the induction/production of RSDE (Dash et al. 2015). Like other carbohydrate degrading enzymes, RSDE production is subjected to catabolite repression by glucose and other readily metabolizable sugars such as fructose, sucrose, etc. (Dash et al. 2015). However, this could be overcome by developing mutants resistant to catabolic repression (Vu et al. 2010). RSDE normally require mesophilic conditions; however, they function optimally below the starch gelatinization temperature (<70 °C) (Robertson et al. 2006), a property that makes them better candidates for industrial processes (Božić et al. 2017).

Supplementation of media with certain metal ions also influences the growth of microorganisms and thereby stimulates or inhibits enzyme production. However, the effector molecules do not hold universal effect, as they may act as stimulators in one organism and inhibitors in other. An example is $LiSO_4$, which was found to be a stimulator of RSDE production by *Bacillus* sp. I-3 (Goyal et al. 2005), but an inhibitor for *Penicillium* sp. X-1 (Sun et al. 2007). Further, physical parameter like agitation regulates oxygen transfer rates which may affect the cell growth and thus influence enzyme production (Liu et al. 2010). Although higher agitation is linked to an increase in biomass and protein production mostly in aerobic bacteria (Kammoun et al. 2008), it may be detrimental for fungal enzyme production, due to shearing (Gupta et al. 2003). Besides physiochemical parameters, fermentation methods, for instance, submerged fermentation (SmF), or solid-state fermentation (SSF) or batch or fed-batch fermentation also influence RSDE production. However, due to the ease in the manipulation of fermentation parameters and upscaling, most of the studies were carried out under submerged conditions. The most important physicochemical parameters affecting the RSDE production are presented in Table 3.1.

3.7 Purification of RSDE

The industrial production of RSDE may not require downstream processing as the purified enzyme is not obligatory for commercial use (Gupta et al. 2003). However, a purified form of the enzyme is a requirement for analyses involving the structure-function relationships, biochemical properties, and for certain industrial applications. The purification strategies employed are strongly dependent on the market demand, processing cost, purity requirement and accessible technology (Sivaramakrishnan et al. 2006). The conventional RSDE purification process involves the separation of cells from the media and precipitation of the enzyme by ammonium sulfate or organic solvents (chilled acetone/ethanol). Subsequently, the concentrated enzyme is subjected to either single or combination of chromatographic techniques such as affinity, ion exchange, hydrophobic interaction, gel filtration,

and/or reverse-phase chromatography (de Souza and e Magalhães 2010). The various strategies employed in the purification of RSDE are listed in Table 3.2.

3.8 Biochemical Properties of RSDE

The properties of microbial enzymes such as temperature and pH optima are mainly growth associated (Gupta et al. 2003). However, secreted enzymes are much more active and stable at temperatures and pH values far above those required for the optimal growth of producers (Haki and Rakshit 2003). The physicochemical properties of purified RSDE from various microorganisms have been studied and the recent progress in this regard is presented in Table 3.3. RSDE are found to be more active near-neutral pH; however, many of them show activity at a wider pH range (pH 4.0–10.0). Most of the RSDE work optimally at mesophilic conditions; however, some can work at temperatures as high as 93 °C (Jana et al. 2013). Further, the size of the RSDE proteins also varies from 55 to 150 kDa, and these proteins either lose or get their activity enhanced in the presence of certain metal ions (Table 3.3). End-product analysis of the enzyme hydrolysates shows that RSDE produce either glucose or maltose or both.

3.9 Raw Starch Hydrolysis Capabilities of RSDE

RSDE can hydrolyze a range of raw starches from different botanical origins such as potato, corn, sweet potato, pea, mung bean, cassava, cocoyam, wheat, rice, water chestnut, horseradish, buckwheat, triticale, jackfruit seeds starch, sweet sorghum, barley, millet, etc. (Table 3.3). However, the extent and type of starch hydrolysis differ with different sources (Jiang et al. 2015). Crystallinity and morphology of the starch granules are some of the factors that determine the type of attack (pore formation vs. peeling) and the extent of hydrolysis (Puspasari et al. 2013). Tuberous starches like potato, cassava and sago have B-type crystalline structure, while cereal starches such as wheat, corn, and rice have A-type structure (Puspasari et al. 2013). B-type starch granules are thicker with larger growth rings and longer average amylopectin branch chain length, making them resistant to enzyme action (Puspasari et al. 2013). Further, there is a mixture of both A- and B-type starches, known as the C-type, found exclusively in smooth-seeded peas, beans, and some cereals (Wei et al. 2010). Raw starch hydrolysis is a heterogeneous reaction, where the enzyme and substrate react at the interface of the starch granules (solid-state) and water (liquid-state). The surface of the starch granules is bonded by water molecules through hydrogen bonding, making the enzyme-substrate interaction difficult (Sun et al. 2010).

Table 3.2 Purification strategy employed for RSDE from various microorganisms

Microorganism	Purification step	Purification fold/yield (%)	Reference
Bacteria			
Alicyclobacillus sp. A4	Ultrafiltration, HiTrap SP XL	/21.5	Bai et al. (2012)
Amphibacillus sp. NM-Ra2	80% ethanol, Q-sepharose FF, ultrafiltration, Superdex™ 75	4.5/15.4	Mesbah and Wiegel (2014)
Anoxybacillus sp. KP1	80% $(NH_4)_2SO_4$	–	Bekler and Güven (2014)
Bacillus sp. UEB-S	Q-Sepharose, Sephacryl S-200	14/51	Maktouf et al. (2013)
B. amyloliquefaciens B-5	Ni^{2+}-NTA resin	–	Liu et al. (2015)
B. licheniformis ATCC 9945a (recombinant)	Superose 12	–	Božić et al. (2013)
B. licheniformis AS08E	Phenyl-Sepharose, Sephacryl S-200	14.5/6.9	Roy and Mukherjee (2013)
B. megaterium VUMB109	80% $(NH_4)_2SO_4$, DEAE cellulose, Sephadex G-100	27.39/38.43	Jana et al. (2013)
B. mojavensis A21	Ultrafiltration, Sephadex G-75, Mono Q	15.3/11.3	Hmidet et al. (2010)
B. subtilis JS 16	$(NH_4)_2SO_4$, DEAE-cellulose, Sephadex-G-100	15.16/4.31	Menon et al. (2014)
B. subtilis S8–18	60% $(NH_4)_2SO_4$, Sephacryl S-200, Q Sepharose	3.7/1.3	Kalpana and Pandian (2014)
B. subtilis AS-S01a	50% $(NH_4)_2SO_4$, acetone, Sephadex G-50	7.5/0.3	Roy et al. (2012)
Exiguobacterium sp. SH3	Sonication, Ni-NTA agarose	–	Rajaei et al. (2015)
Geobacillus sp. IIPTN	Ultrafiltration, macro prep high S, CHT type 1 ceramic hydroxyapatile binding	82/31	Dheeran et al. (2010)
Geobacillus sp. 4j	60% $(NH_4)_2SO_4$, Ni^{2+}-NTA resin	–	Jiang et al. (2015)
G. thermoleovorans	Ni^{2+}-NTA resin	–	Mehta and Satyanarayana (2014)
G. thermoleovorans NP33	Acetone precipitation, Resource™ Q, Sephacryl™ S-200	35.7/19.07 (amylase) 33.17/17.68 (pullulanase)	Nisha and Satyanarayana (2014)
G. thermoleovorans subsp. *Stromboliensis* subsp. nov. (strain Pizzo^T)	80% $(NH_4)_2SO_4$, Q-Sephaose F. F., Superdex S 200, ultrafiltration	68.9/9.2	Finore et al. (2011)
Halolactibacillus sp. SK71	70% $(NH_4)_2SO_4$, Q-Sepharose, Superdex G-75	9.5/21.8	Yu and Li (2014)

(continued)

Table 3.2 (continued)

Microorganism	Purification step	Purification fold/yield (%)	Reference
Halorubrum xinjiangense	80% ethanol, starch affinity, ultrafiltration, Mono Q™	–	Moshfegh et al. (2013)
Lactobacillus fermentum 04BBA19	65% $(NH_4)_2SO_4$, dialysis	–	Fossi et al. (2011)
Unknown marine bacterium (α-amylase)	Ni^{2+}-NTA resin	–	Lei et al. (2012)
Fungi/yeast			
Aspergillus sp. MZA-3	60% $(NH_4)_2SO_4$	–	Moshi et al. (2015)
A. flavus	70% $(NH_4)_2SO_4$, dialysis	15.64/32.67	Adeyanju et al. (2014)
Aureobasidium pullulans NRRL 12974	Ultrafiltration, Sephacryl S-100 HR, Toyopearl DEAE-650M	–	Li et al. (2011)
Rhizopus oryzae WCS-1	77% $(NH_4)_2SO_4$	–	Jang et al. (2015)
Talaromyces stipitatus	QuixStand, diafiltration, DEAE-Sepharose FF, SP-Sepharose 4 FF, HiLOAD Superdex 75, 200	13/5 (amylase) 4/60 (glucoamylase)	Xiao et al. (2014)

3.10 Molecular Characterization of RSDE

Molecular cloning and expression of a particular enzyme are primarily carried out for their hyper-production, while protein/enzyme engineering is done to integrate desired properties into the appropriate gene (Singh et al. 2013). On the protein side, the desired properties for RSDE may include an increase in thermostability, extension of pH profile, Ca-independence, ability to degrade various raw starch, activity at high starch concentration, protease resistance, etc., while on the production side, removal of catabolite repression or co-expression of different amylolytic enzymes is desired (Sivaramakrishnan et al. 2006). However, molecular cloning and protein engineering strategies also have some limitations. For the successful expression/overexpression of a recombinant protein, several factors including the selection of expression host, vectors, promoters, integration site of the foreign gene, gene copy number, regulatory proteins, and genetic stability are critical (Kumar and Satyanarayana 2009).

3.10.1 Cloning and Expression of RSDE

Cloning of RSDE genes and their expression in a homologous or heterologous host is crucial for enzyme engineering and hyperproduction at an industrial scale (Nisha

Table 3.3 Biochemical properties of some RSDEs and their raw starch digesting capabilities

Organism	Molecular weight (kDa)	pH optima/ stability	Temperature optima/ stability (°C)	Enhancers	Inhibitors	Raw starch digestion	Products	Reference
Bacteria								
Aeromonas salmonicida ssp. *salmonicida* A449	55	7.0	40	Ca^{2+}, Co^{2+} and Cu^{2+}	EDTA	Rice, corn, wheat, potato, mung, pea	Glucose, maltose, and maltotriose	Peng et al. (2015)
Alicyclobacillus sp. A4	70	4.2/ 3.8–5.4	75/75	Co^{2+}, Mn^{2+}, β-mercaptoethanol	Cr^{3+}, Cu^{2+}, Fe^{3+}, Pb^{2+}, Hg^{2+}, Ag^{+}, SDS	Potato, corn, sweet potato, pea, mung bean	–	Bai et al. (2012)
Amphibacillus sp. NM-Ra2	50	8.0/ 7.0–8.5	54/45–60	K^{+}, Ca^{2+}, SDS, Tween 80, DTT	Mg^{2+}, Cu^{2+}, β-mercaptoethanol, EDTA, urea	Wheat, corn, potato	Maltose and maltotriose	Mesbah and Wiegel (2014)
Anoxybacillus sp. KP1	–	8.0/ 6.0–10.0	60/60–70	Mn^{2+}	Hg^{2+}, SDS	Wheat, rice, corn	–	Bekler and Güven (2014)
Bacillus sp. UEB-S	130	5.0/ 4.0–9.0	70/30–90	–	–	Wheat, barley, and millet	Glucose, maltose	Maktouf et al. (2013)
B. amyloliquefaciens B-5	66	5.0/ 4.0–6.0	70/55–60	K^{+}, Ca^{2+}, Mg^{2+}	Mn^{2+}, Fe^{2+}, Fe^{3+}, EDTA	Wheat, corn, potato, sweet potato	Glucose and maltose	Liu et al. (2015)
B. aquimaris MKSC 6.2	70	6.5	37	–	–	Cassava, corn	Glucose, maltose, maltotriose, maltotetraose	Puspasari et al. (2013)
B. licheniformis ATCC 9945a	55.2	6.5	90	–	–	Wheat, potato, horseradish, corn	–	Božić et al. (2013)
B. licheniformis AS08E	55	10/6.0–12.0	80/40–90	–	EDTA, 4-BPB, Hg^{2+}	Jackfruit seeds starch	Maltose, maltotriose	Roy et al. (2015)

B. licheniformis AT70	85	8.0/ 6.0–9.0	60/30–60	Mn^{2+}	Mg^{2+}, Ag^{+}	Corn	–	Afrisham et al. (2016)
B. megaterium VUMB109	150	7.75/–	93/–	Mg^{2+}, Sn^{2+}, K^{+}	Hg^{2+}, Cu^{2+}, Fe^{3+}, Mn^{2+}, Zn^{2+}	Rice, potato, wheat, and corn	Maltotriose, maltopentaose	Jana et al. (2013)
B. mojavensis A21	58	6.5/ 4.0–9.0	80/30–90	–	Hg^{2+}, Zn^{2+}, EDTA	Potato, wheat, corn	Maltooligosaccharides	Hmidet et al. (2010)
B. subtilis S8–18	57	6.0/ 4.0–12.0	60/40–60	–	Hg^{2+}, Zn^{2+}, SDS	Corn, potato, wheat	Glucose and maltose	Kalpana and Pandian (2014)
B. subtilis Strain JS-16	99/87	9.0/ 8.0–9.5	50/20–80	Fe^{2+}, SDS	Hg^{2+}	Wheat, corn	–	Menon et al. (2014)
B. subtilis AS01a	69	6.0/ 5.0–9.0	70/40–80	–	Fe^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}	Potato, wheat, rice	Glucose and maltose	Roy et al. (2013)
Exiguobacterium sp. SH3	110	8.5/ 5.0–11.0	45/40–50	Mn^{2+}, β-mercaptoethanol, DTT	Cu^{2+}, Zn^{2+}	Starch, rice, potato	Maltose, maltotriose, maltotetraose	Rajaei et al. (2015)
Geobacillus sp. IIPTN	97	5.0/ 4.5–9.5	80/40–120	Mn^{2+}, Ca^{2+}, Ba^{2+}, Co^{2+}, Na^{+}, Fe^{3+}, K^{+}	Mg^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, EDTA	Cassava, corn, soluble starch, tapioca root, sweet sorghum	Maltose, maltotriose, glucose	Dheeran et al. (2010)
Geobacillus sp. 4j	62	5.5/ 4.5–7.0	65/55–80	Ca^{2+}, K^{+}, Na^{+}, Mg^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}, Fe^{2+}, Fe^{3+}	Mn^{2+}, Cu^{2+}, and Ag^{+}	Corn, wheat, cassava, sweet potato, pea	Maltose	Jiang et al. (2015)
G. Thermoleovorans	56	7.0	60/40–70	Glycerol	SDS, Mn^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Al^{3+}, *N*-bromosuccinimide	Corn, wheat, water chestnut, tapioca	G1, G2, G3, G4	Mehta and Satyanarayana (2014)
G. thermoleovorans NP33	105	7.0/ 6.0–9.0	80/60–90	Zn^{2+}, Mn^{2+}	Cu^{2+}, EDAC, and Woodward's reagent K	Wheat, rice, corn, water chestnut	Maltose, maltotriose, maltotetraose glucose	Nisha and Satyanarayana (2014)

(continued)

Table 3.3 (continued)

Organism	Molecular weight (kDa)	pH optima/ stability	Temperature optima/ stability (°C)	Enhancers	Inhibitors	Raw starch digestion	Products	Reference
Geobacillus (K1C)	59	6.0/ 5.0–7.0	80/70–80	–	Cu^{2+}, Pb^{2+}, and Hg^{2+}, EDTA, β-mercaptoethanol, DTT, and PMSF	Rice, wheat, corn, insoluble potato	–	Sudan et al. (2018)
G. thermoleovorans subsp. *stromboliensis* subsp. *nov*	58.0	5.6/ 4.0–10.0	70/30–100	–	N-Bromosuccinimide, p-hydroxymercuribenzoate, EDTA	Raw corn starch, wheat starch	Maltose, maltotriose, maltooligosacharides	Finore et al. (2011)
Halolactibacillus sp. SK71	78.5	8.0/ 7.0–12.0	70/50–90	–	–	Corn, potato, wheat, rice	Glucose	Yu and Li (2014)
Laceyella sacchari LP175	–	7.0	50	–	–	Cassava	–	Lomthong et al. (2015)
Lactobacillus fermentum 04BBA19	–	4.0–7.0/ 4.0–8.0	60–70/30–100	Ca^{2+}, Fe^{2+}, Fe^{3+}, Na^{+}, and Mg^{2+}	Cu^{2+}, EDTA	Cassava, potato, cocoyam	–	Fossi et al. (2011)
Microbacterium aurum B8.A	95	–	37/–	–	–	Wheat, tapioca, potato	Maltose, maltotriose, maltooligosaccharides	Sarian et al. (2012)
Streptomyces badius DB-1	57	6.0/ 6.0–9.0	50/–	Ca^{2+}, Mg^{2+}	Zn^{2+}, Cu^{2+}, Hg^{2+}, EDTA, EGTA, NBS, Woodward's reagent K	Wheat, sago, rice, corn, water chestnut	Glucose, maltose, maltotriose, maltotetraose	Shivlata and Satyanarayana (2017)
Fungi/yeast								
Aspergillus spp.	–	5.0/–	45/–	K^{+}, Ca^{2+}	Mg^{2+}, EDTA	Cassava, garri, tapioca	–	Avwioroko (2015)
Aspergillus sp.	–	5.5	50	–	–	Cassava	Maltose, maltotriose, glucose	Moshi et al. (2015)
A. carbonarius	–	5.0	30	–	–	Potato	–	Nwagu et al. (2012)
A. flavus	–	5.0/–	70/–	–	–	Cassava	–	Adeyanju et al. (2014)

A. tubingensis	110–150	4.0/ 3.0–5.0	60	–	–	Corn	–	Viktor et al. (2013)
A. tubingensis	90	4.5	70	–	–	Corn	–	
Aureobasidium pullulans NRRL 12974	66	4.5/ 3.0–6.0	60/30–65	–	–	Potato	–	Li et al. (2011)
Penicillium sp. GXU20	–	4.5	50	–	–	Cassava, corn, rice, potato, sweet potato, buckwheat	–	Lin et al. (2011)
P. oxalicum GXU20	75.4	4.5/ 2.0–10.5	65/30–60	Mn^{2+} and Fe^{2+}	Ag^{+}, Cu^{2+}, SDS, EDTA	Cassava, corn, rice, potato, sweet potato, buckwheat		Xu et al. (2016)
Rhizopus oryzae WCS-1	–	4.0	55	Na^{+}, Ca^{2+}	Hg^{2+}, Ag^{2+}	Wheat	L-glucose	Jang et al. (2015)
Talaromyces stipitatus	70	5.0/ 4.0–6.0	50–60/45–65	Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+} (1 mM)	Fe^{3+}, Co^{2+} (5 mM), Mg^{2+} (10 mM)	Triticale, potato	Glucose	Xiao et al. (2014)
T. stipitatus	66	5.0/ 4.0–6.0	50/45–65	Mn^{2+}, Fe^{3+}, Co^{2+} (1 mM)	–	Triticale, potato		
Archaea								
Halorubrum xinjiangense	60	8.5/ 8.0–9.0	70/30–90	Ca^{2+}	Fe^{2+}, Fe^{3+}, Hg^{2+}, Al^{3+}, Zn^{2+}	Wheat, corn, potato	Maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose	Moshfegh et al. (2013)

and Satyanarayana 2013). A large number of RSDE encoding genes from bacterial and fungal genomes have been cloned and expressed (Table 3.4). The most preferred bacterial host for the heterologous expression of RSDE genes is *Escherichia coli* (Parashar and Satyanarayana 2016), due to the ease in genetic manipulation and faster growth (Roy et al. 2015). For the expression of eukaryotic RSDE genes, where posttranslational modification of the product takes place, the preferred choice is either *Pichia pastoris* (Li et al. 2011) or *Saccharomyces cerevisiae* (Favaro et al. 2015). However, some other eukaryotic expression systems such as *Kluyveromyces marxianus* and *Yarrowia lipolytica* have also been used (Wang et al. 2014).

3.10.2 Engineering for Improvement of RSDE Activity

Enzyme engineering is a powerful technique to modify or integrate desired properties in the appropriate RSDE gene to improve its catalytic efficiency (Sivaramakrishnan et al. 2006). Viktor et al. (2013) attempted to co-express α-amylase (amyA) and glucoamylase (glaA) from *A. tubingensis* T8.4 in *S. cerevisiae* for improving raw starch utilization and direct bioethanol production. A promising strategy to increase the efficacy of biocatalysts is co-displaying of enzymes on the yeast cell surface. A raw starch digesting α-amylase from *Streptococcus bovis* and glucoamylase from *Rhizopus oryzae* were co-displayed on the cell surface of *S. cerevisiae* for repeated fermentation of raw starch to ethanol (Yamakawa et al. 2012). Inokuma et al. (2015) developed gene cassettes for the cell surface display of *S. bovis* α-amylase and *R. oryzae* glucoamylase using the *S. cerevisiae* SED1 promoter and two different glycosylphosphatidylinositol (GPI)-anchoring regions. These cassettes were integrated in different combinations into the genome of ethanologenic *S. cerevisiae*. The highest ethanol yield from raw starch was achieved in the strain harboring one α-amylase gene cassette carrying the SED1-anchoring region and two glucoamylase gene cassettes carrying the SED1-anchoring region (Inokuma et al. 2015). The fusion of a barley α-amylase gene with *Lentinula edodes* glucoamylase genes and their integration into the chromosome of *S. cerevisiae* resulted in a recombinant strain that could efficiently hydrolyze raw starch (Görgens et al. 2015). Parashar and Satyanarayana (2016) engineered the α-amylase (Ba-amy) of *B. acidicola* by fusing it with the gene encoding partial N- and C-terminal regions of thermostable α-amylase gene of *G. thermoleovorans* (Gt-amy). The resulting chimeric enzyme (Ba-Gt-amy) showed a marked increase in catalytic efficiency. Similarly, the truncation of the N1 domain of raw starch digesting amylopullulanase (gt-apuΔN) from *G. thermoleovorans* NP33 led to the enhancement of thermostability and specific activity as compared to native amylopullulanase (gt-apu) (Nisha and Satyanarayana 2015).

Table 3.4 RSDEs cloning host, optimum temperature, pH, and substrate

Source	Host	Temperature (°C)	pH	Starch source	Reference
Aeromonas salmonicida ssp. *salmonicida* A449	*E. coli*	40	7	Rice, wheat, and mung starches	Peng et al. (2015)
Aspergillus oryzae	*Kluyveromyces marxianus*	42	6	Raw corn starch	Wang et al. (2014)
A. tubingensis	*Saccharomyces cerevisiae*	55/65	4	Raw corn starch	Viktor et al. (2013)
Aureobasidium pullulans NRRL 12974	*P. pastoris*	60	4.5	Raw potato starch	Li et al. (2011)
Bacillus acidicola/G. thermoleovorans	*E. coli*	60/70	4/5	Raw corn starch	Parashar and Satyanarayana (2016)
B. amyloliquefaciens	*E. coli*	50	5	Raw rice starch	Gangadharan et al. (2010)
B. amyloliquefaciens	*E. coli*	70	5	Potato, sweet potato, wheat, and corn starch	Liu et al. (2015)
B. aquimaris MKSC 6.2	*E. coli*	37	6.5	Raw cassava, corn starch	Puspasari et al. (2013)
B. licheniformis AS08E	*E. coli*	80	10	Raw jack fruit seed starch	Roy et al. (2015)
B. licheniformis ATCC 9945a	*E. coli*	90	6.5	Wheat, potato, horseradish, corn	Božić et al. (2013)
B. subtilis PY22	*P. pastoris*	60	7.0	MM starch	Karakaş et al. (2010)
B. subtilis AS01a	*E. coli*	70	6.0	Wheat, potato, and rice raw starch	Roy and Mukherjee (2013)
Debaryomyces occidentalis	*S. cerevisiae*	40	5.5	Raw starch	Kim et al. (2011)
Geobacillus sp.	*E. coli*	60–65	5.5	Raw starch	Jiang et al. (2015)
G. thermoleovorans	*E. coli*	80	7	Raw sago starch	Nisha and Satyanarayana (2013)
Microbacterium aurum B8.A	*E. coli*	37	6.8	Potato and wheat starch	Valk et al. (2015)
Neurospora crassa	*S. cerevisiae*	40	3.0	Raw starch	Guo et al. (2011)
Penicillium oxalicum GXU20	*P. pastoris*	65	4.5	Raw corn and cassava flour	Xu et al. (2016)
Saccharomycopsis fibuligera	*S. cerevisiae*	42	4.5	Raw corn starch	Favaro et al. (2015)
Streptococcus bovis	*S. cerevisiae*	37	–	Raw corn starch	Yamakawa et al. (2012)

(continued)

Table 3.4 (continued)

Source	Host	Temperature (°C)	pH	Starch source	Reference
Talaromyces stipitatus	*E. coli*	50	5.0	Raw starch	Xiao et al. (2014)
Thermotoga petrophila	*E. coli*	22	7.0	Corn and rice starch	Zafar et al. (2016)
Thermobifida fusca	*Pichia pastoris*	60	7	Raw sago starch	Yang et al. (2010a)
T. fusca	*Yarrowia lipolytica*	60	7	Raw sago starch	Yang et al. (2010b)

3.11 Applications of RSDE in Biofuel Production

3.11.1 Bioethanol

The overdependence of fossil fuels for energy is not sustainable due to its gradual depletion, increasing cost, and contribution to global warming. This has led to a gradual shift from fossil fuels to renewable energy resources. Biofuel production from renewable biomass is rapidly gaining momentum due to clean, eco-friendly, sustainable, and cost-competitive energy sources. Besides, it does not compete with food production and aids in effectively managing agricultural waste (Boodhun et al. 2017). Ethanol is one of the most important biofuels produced from different renewable feedstock, and its global production has increased by 100–500% in recent years (http://globalrfa.org). However, a significant amount of bioethanol is being produced from starch-based feedstocks (Zyl et al. 2012). The economic viability of starch-based ethanol production is limited though due to the energy-intensive starch gelatinization step, and the costs associated with enzymatic liquefaction and saccharification. The impetus is therefore on exploiting RSDE-producing microorganisms that can bypass these cost-intensive processes, thereby assisting in reducing the total energy capital and operational costs (Cinelli et al. 2015).

Although studies pertaining to raw starch hydrolysis have been carried out for a long time, reports on the multifarious applications of the saccharified product have gained momentum recently. The non-conventional simultaneous saccharification and fermentation (SSF) process of bioethanol production involves the preparation of raw starch suspension, cold hydrolysis using RSDE followed by fermentation steps (Castro et al. 2011). Various microbes have been explored for their ability to produce ethanol using RSDEs (Table 3.5). Ueda (1981) observed a marked improvement in bioethanol production using raw cassava root homogenate and enzyme extract from *Aspergillus awamori* NRRL 3112 and *A. niger*. Different strains of *A. niger* are known to hydrolyze raw corn starch (Han and Steinberg 1987) and raw sago starch (Pranamuda et al. 1995) to produce ethanol. Moshi et al. (2015) reported that *Aspergillus* sp. MZA-3 when co-cultured with *S. cerevisiae* at 30 °C, produced ethanol using inedible wild cassava flour with 91% of theoretical yield and 84% efficiency. Attempts to co-immobilize *A. awamori* with *Zymomonas mobilis* (A-Z system) and *Rhizopus japonicus* (R) with *Z. mobilis* (R-Z system) produced higher titers of amylase and glucoamylase, respectively, to digest raw rice starch (Lee and Parulekar 1993). A higher ethanol yield (96%) and reduction in fermentation time were achieved when all the three microorganisms were co-immobilized (A-R-Z system). The white-rot fungus *Trametes hirsuta* produced 9.1 g/L ethanol using raw starch, which corresponds to 89.2% of the theoretical yield (Okamoto et al. 2011).

Mutations using γ-irradiation, ultraviolet light, and *N*-methyl-*N*′-nitrosoguanidine treatments have been used to improve ethanol yield. Rajoka et al. (2004) reported a 2.5-fold improvement in glucoamylase production from a γ-irradiated strain of *A. niger*. The mutant-derived glucoamylases hydrolyzed raw maize starch with improved ethanol productivity (1.42 g/L/h) than the parent strain

Table 3.5 Bioethanol production using RSDE/RSDE producing organism

Raw starch source	Fermentation conditions	Fermentation enzymes/ agents	Ethanol conversion (%)	Reference
Simultaneous saccharification and fermentation				
Babassu flour	32 °C, pH 4.8, 48 h	*S. cerevisiae/A. awamori* extract	83	Cinelli et al. (2014)
Cassava	40 °C, pH 4.0, 36 h	rPoGA15A and α-amylase/ *S. cerevisiae*	93.5	Xu et al. (2016)
Cassava (wild inedible)	Pretreatment (30 °C, 24 h) with MZA-3/ 32 °C, 84 h	*S. cerevisiae/Aspergillus* sp. *MZA-3*	14	Moshi et al. (2015)
Cassava (chips)	Pretreatment (50 °C, 6 h), 42 °C, 48 h, 250 rpm	*R. microsporus* TISTR 3531/ commercial amylase/ *Kluyveromyces marxianus* DMKU-KS07	88	Trakarnpaiboon et al. (2017)
Cassava	40 °C, pH 4.0, 48 h	*P. oxalicum* extract/ *S. cerevisiae*	92	Lin et al. (2011)
Cassava	37 °C, pH 4.0, 85 h	*A. kawachii* extract/yeast	92.3	Sugimoto et al. (2012)
Cassava	35 °C, pH 4.5, 96 h	*Rhizopus* koji	72.3–83.5	Fujio et al. (1985)
Cassava	35 °C, pH 4.5–5.0, 288 h	*Rhizopus* koji	74.5–85.5	Fujio et al. (1984)
Corn	30 °C, 48 h	*Rhizopus* sp. extract/ *S. cerevisiae*	94.5	Wang et al. (2007a)
Corn	40 °C, pH 4.0, 48 h	rPoGA15A and α-amylase/ *S. cerevisiae*	95.1	Xu et al. (2016)
Corn	30 °C, pH 3.5, 96 h	*Aspergillus* sp. extract/ *S. cerevisiae*	92.7–94.0	Vu et al. (2010)
Corn	27 °C, pH 4.2, 96 h	*S. cerevisiae/RHS BPX™ enzyme*	18.5	Lamsal et al. (2011)
Corn	30 °C, pH 5.0, 120 h	*Chalara paradoxa* extract/ *S. cerevisiae*	63.5–86.8	Mikuni et al. (1987)
Corn	30 °C, pH 5.0, 120 h	*Chalara paradoxa* extract/ *S. sake*	81.1–92.1	Mikuni et al. (1987)
Corn	30 °C, pH 4.1–4.3, 72 h	*A. niger* amylases/yeast	95.9	Han and Steinberg (1987)
Corn	30 °C, pH 4.0, 72 h	STARGEN 001/*S. cerevisiae*	88.4	Wang et al. (2007b)
Corn	32 °C, pH 3.7, 70 h	STARGEN 001/*S. cerevisiae*	91.3	Adams et al. (2012)
Corn	32 °C, pH 3.7, 70 h	STARGEN 002/*S. cerevisiae*	85.9	Adams et al. (2012)
Corn	35 °C, pH 5.0, 72 h	STARGEN 001/*S. cerevisiae*	83.4	Białas et al. (2010)

(continued)

Table 3.5 (continued)

Raw starch source	Fermentation conditions	Fermentation enzymes/agents	Ethanol conversion (%)	Reference
Sago	40 °C, pH 3.5	*S. cerevisiae/A. niger*	70.5	Pranamuda et al. (1995)
Wheat bran	30 °C, 240 h, 150 rpm	Engineered *S. cerevisiae* secreting glucoamylase (TLG1) and α-amylase (SFA1)/cellulase cocktail	88	Cripwell et al. (2015)
Wheat-rye bread	35 °C, pH 4.5, 48 h	STARGEN 002/Neutrase/ *S. cerevisiae*	80	Pietrzak and Kawa-Rygielska (2014)
Consolidated bioprocessing				
Cassava	42 °C, pH 4.8–5.6, 96 h	Engineering *K. marxianus*	78.3	Wang et al. (2014)
Corn	30 °C, 72 h	*S. cerevisiae* codisplaying *Rhizopus oryzae* glucoamylase and *Streptococcus bovis* α-amylase	86.5	Shigechi et al. (2004)
Corn	30 °C, 240 h	*Saccharomyces cerevisiae* strains expressing α-amylases and glucoamylases/STARGEN 002	90.19	Sakwa et al. (2018)
Corn	30 °C, 20 batches (one batch for 24 h)	*S. cerevisiae* displaying amylolytic enzymes	76.6	Yamakawa et al. (2012)
Corn	30 °C, 120–240 h	Engineered *S. cerevisiae*	61–80	Viktor et al. (2013)
Corn	30 °C, 240 h	Engineered *S. cerevisiae* secreting glucoamylase (TLG1) and α-amylase (SFA1)	55	Favaro et al. (2015)
Sorghum	30 °C, 240 h	Engineered *S. cerevisiae* secreting glucoamylase (TLG1) and α-amylase (SFA1)	62	Favaro et al. (2015)
Triticale	30 °C, 240 h	Engineered *S. cerevisiae* secreting glucoamylase (TLG1) and α-amylase (SFA1)	73	Favaro et al. (2015)

(1.23 g/L/h). Similarly, mutant *Aspergillus* sp. XN15 produced 19.4-times higher levels of RSDE than the parent strain. Simultaneous saccharification and fermentation using RSDE and *S. cerevisiae* yielded 15.82% and 20.75% (v/v) ethanol from 25.73% and 35.78% (w/v) raw rice corn starch slurry, respectively (Vu et al. 2010).

One of the major limitations in ethanol production using RSDE during non-conventional SSF is their less favored enzyme kinetics, due to below-optimum enzyme activities at high substrate loading and moderate operational temperatures. Apart from *Arxula adeninivorans* (Büttner et al. 1992), *Endomycopsis fibuligera* (Reddy and Basappa 1993), and *Scheffersomyces shehatae* JCM 18690 (Tanimura et al. 2015), there are not many yeasts that can directly produce ethanol from starch. Though *S. cerevisiae* remains the preferred yeast for industrial production of ethanol due to high yield, productivity, and tolerance to ethanol, it is unable to hydrolyze complex substrates (Zyl et al. 2012). It is therefore imperative to clone and overexpress RSDE in industrial strains of *S. cerevisiae* for simultaneous starch hydrolysis and ethanol production. Reports are available on the expression of amylase genes from several microbial sources in *S. cerevisiae.* However, co-expression of α-amylases and glucoamylases ensures enhanced hydrolysis of raw starch due to the synergistic action of the expressed enzymes which results in higher ethanol production rates. A cell-surface engineered *S. cerevisiae* displaying both glucoamylase and α-amylase was recycled (23 times) without loss of enzyme activity under high raw corn starch load (100 g/L) (Yamakawa et al. 2012). Higher starch hydrolysis rates and ethanol yields were achieved with yeasts producing extracellular enzymes (Liao et al. 2010). Adsorption of RSDE to the solid granular raw starch is a rate-limiting step; however, as the reaction progresses, small cavities are formed facilitating the diffusion of RSDE into pores and channels (Cinelli et al. 2015). Generally, ethanol production using RSDE is low in comparison to the process involving starch gelatinization due to the mass transfer limitations during heterogeneous catalysis. Khaw et al. (2007) showed the importance of surface contact between starch granules and yeast cells with cell wall anchored α-amylase and glucoamylase for enhanced ethanol production rate.

Engineering and overexpression of RSDE are advantageous; however, overexpression of RSDE in ethanologenic yeast strain using yeast episomal plasmid (YEp) vectors is disadvantageous. This is due to the risk associated with mitotic instability of plasmids and the continuous need for selectable markers for maintenance, while expression in yeast integrative plasmids (YIp) is not suitable due to just one copy of the gene (Görgens et al. 2015). Additionally, the haploid strains are unsuitable due to low tolerance levels. Presently δ-sequences of the Ty retrotransposon and rDNA are preferred as targets for RSDE integration as it ensures polyploidization and overexpression of the amylolytic proteins. This strategy was used to construct a tetraploid strain harboring *S. bovis* α-amylase and *R. oryzae* glucoamylase/α-agglutinin fusion protein genes (Yamada et al. 2010). The tetraploid strain showed 1.5- and 10-fold improvement in α-amylase and glucoamylase activities, respectively, producing 70 g/L of ethanol from 150 g/L of raw corn starch in 72 h. Similarly, a polyploid *S. cerevisiae* strain co-expressing glucoamylase from *A. awamori* and amylase from *Debaryomyces occidentalis* generated 80.9 g/L ethanol from 200 g/L raw corn starch (Kim et al. 2011). Other amylolytic *S. cerevisiae* Mnuα1 strain, developed by expressing *A. tubingensis* α-amylase and glucoamylase genes produced 70 g/L ethanol from 200 g/L raw corn starch (Viktor et al. 2013). Codon-optimized variants of *Thermomyces lanuginosus* glucoamylase

and *Saccharomycopsis fibuligera* α-amylase genes were δ-integrated into *S. cerevisiae* to produce 64 g/L ethanol from raw corn starch (Favaro et al. 2015). Engineered amylolytic yeast strains look promising for RSDE-mediated consolidated bioprocessing of raw starch; however, their industrial-scale implementation requires further research (Görgens et al. 2015).

3.11.2 Biobutanol

Among the alternative fuels, biobutanol has shown promise as its properties are much similar to gasoline (Ranjan and Moholkar 2012). In comparison to ethanol, biobutanol has a longer carbon chain length, higher volatility, polarity, combustion value, octane rating (Knoshaug and Zhang 2009), and less corrosive (Oudshoorn et al. 2009). It can be used as a substitute for gasoline without altering the current vehicles or engines and can be blended at any ratio with gasoline, unlike ethanol (Cheng et al. 2012). It has fewer ignition problems and an engine running on butanol should be easier to start in cold weather, as the heat of vaporization of butanol is less than half of ethanol (Sullivan et al. 2010). With the fluctuation of global petroleum prices and the uncertainty of future raw oil supply, butanol production by fermentation has attracted wide interest (Lépiz-Aguilar et al. 2013).

The most favorable biobutanol-producing strains belong to *Clostridium* species (Kumar and Gayen 2011), e.g., *C. acetobutylicum, C. beijerinckii, C. saccaroperbutylacetonicum, C. saccharoacetobutylicum, C. aurantibutyricum, C. pasteurianum, C. sporogenes, C. cadaveris, and C. tetanomorphum*, because of its unique characteristics such as high sugar uptake, high specific butanol production, and high biobutanol/ethanol tolerance in acetone-butanol-ethanol (ABE) fermentation (Thirumalai Vasan et al. 2011). However, commercial biobutanol production is limited by the end-product toxicity. Even the most tolerant strains can rarely tolerate more than 2% butanol (Kumar and Gayen 2011).

Biobutanol production has been evaluated with various substrates such as maltodextrin, wheat straw hydrolysate, corn-derived waste, packing peanuts, and soy molasses (Lépiz-Aguilar et al. 2013). However, biobutanol production from lignocelluloses is less cost-effective due to complicated material processing (Yang et al. 2017). Thus, starchy materials are widely used as raw materials as they are abundant and cost-effective (Thang et al. 2010). Unlike bioethanol, conventional conversion of starch to biobutanol requires a gelatinization step followed by fermentation, as solventogenic *Clostridia* can utilize directly gelatinized starches to produce solvents, without enzymatic pretreatment (Thang and Kobayashi 2014). However, RSDE can act directly on raw starches and simplify the process, thereby reducing the energy requirement of biobutanol production by 10–20% (Thang and Kobayashi 2014). Hence, biobutanol production from starchy material directly by an ethanologenic strain harboring RSDE deemed superior to the conventional process. Table 3.6 shows some of the cases where RSDE have been applied for biobutanol production.

Table 3.6 Cases of biobutanol production using RSDE/RSDE producing organism

Raw starch source	Fermentation enzymes/agents	Butanol (g/L)	Butanol productivity (g/L/h)	Reference
Cassava	Granular starch hydrolyzing enzyme (Stargen 001)/ *C. saccharoperbutylacetonicum*N1–4	17.5	0.24	Thang and Kobayashi (2014)
Corn	Granular starch hydrolyzing enzyme (Stargen 001)/ *C. saccharoperbutylacetonicum*N1–4	17.5	0.36	Thang and Kobayashi (2014)
Wheat	Granular starch hydrolyzing enzyme (Stargen 001)/ *C. saccharoperbutylacetonicum*N1–4	17.8	0.29	Thang and Kobayashi (2014)
Cassava	Coculture *of Bacillus subtilis* WD 161/*C. butylicum* TISTR 1032	6.7	0.09	Tran et al. (2010)
Cassava flour	*Clostridium.* sp. strain BOH3	17.8	0.25	Li et al. (2015)
Potato starch (waste)	*C. acetobutylicum* immobilized on calcium alginate-polyvinyl alcohol (PVA)-boric acid beads	15.3	–	Kheyrandish et al. (2015)

The first report on the direct fermentation of native starches using RSDE in acetone-butanol-ethanol (ABE) fermentation was reported by Thang and Kobayashi (2014) using granular starch hydrolyzing enzyme (Stargen 001) and *Clostridium saccharoperbutylacetonicum* N1–4. They achieved a butanol concentration of 17.5 ± 0.4 to 17.8 ± 0.3 g/L after 72 h fermentation using cassava, corn, and wheat starches. However, *Clostridium* sp. does not utilize starch much due to the low activity of the amylases produced by them. Further, starch pretreatment by either enzymes or acids has negative feedback effects (Tran et al. 2010). A mixed culture of *C. butylicum* TISTR 1032 and amylolytic *B. subtilis* WD 161 was applied to enhance ABE production from starch and achieved a butanol concentration of 6.7 g/L from cassava raw starch after 72 h fermentation (Tran et al. 2010). Li et al. (2015) manipulated the cofactor [e.g., Ca^{2+} and NAD/(P)H] levels to increase the efficacy of biobutanol production by *Clostridium* sp. strain BOH3 from raw cassava flour. Recent trends in ABE fermentation focus on reducing the production cost using cheaper, abundant, and sustainable feedstocks, such as wastes and agricultural residues. Kheyrandish et al. (2015) explored waste starch from potato factories for biobutanol production using immobilized cells to reduce the negative impact of product inhibition and improve productivity and final butanol concentration. Though several attempts were made to utilize gelatinized starch or supplementing with cellulosic hydrolysates for ABE fermentation, very few efforts have been made toward directly utilizing raw starch, due to low productivity and product inhibition (Kumar and Gayen 2011).

3.11.3 Biohydrogen

Biohydrogen is the third subset of biofuel, and among biofuels, it has the highest potential as a future energy carrier because of its reduced emission of air pollutants and greenhouse gases. Hydrogen fuel is considered a clean and green biofuel, as its only combustion product is water, and does not release any toxic gases or CO_2 into the environment (Boodhun et al. 2017). Biohydrogen is produced mainly by bio-photolysis using algae and cyanobacteria, photo-decomposition of organic compounds by photosynthetic bacteria and dark fermentation from organic compounds with anaerobes (Show and Su 2011). Most of the hydrogen-producing bacteria include *Enterobacter, Bacillus,* and *Clostridium* species, however, most of them cannot use starch directly (Vendruscolo 2015). Besides, the major hurdle for commercial biohydrogen production is the high production costs; thus, there is a great demand for developing alternative and economically feasible strategies (Vendruscolo 2015). Starch being abundantly available and could easily be hydrolyzed to simple sugars, which in turn be converted to organic acids and then to hydrogen proves to be an effective, low-cost substrate for biohydrogen production (Vendruscolo 2015). Although there are many reports on the utilization of starch from rice, corn, cassava, wheat, sorghum, potato, sago, or their wastes for biohydrogen production, very few reports are available on the utilization of raw starch directly for biohydrogen production using RSDE or producing microbes (Boodhun et al. 2017). Wang et al. (2017) employed raw cassava starch for biohydrogen production and obtained a maximum yield of 1.72 mol H_2/mol glucose via dark fermentation, using a mixed-culture of *B. cereus* and *Brevumdimonas naejangsanensis*. Bao et al. (2012) also explored mixed culture strategies for hydrogen production from raw corn starch and obtained significantly high production levels (1.04 mol H_2/mol glucose) as compared to a single culture. Although various efforts are being made to bring down biohydrogen production costs, it remains expensive than other biofuels (Boodhun et al. 2017). Thus, utilizing starch-containing biomass as an alternative substrate for biohydrogen production would be more efficient and economical by employing RSDE or RSDE producing microbes.

3.12 Conclusions and Outlook

RSDE still represent a hot topic due to the global fuel crisis, environmental concerns, and requirement for value addition to starchy crops. The global quest to find an alternative process for sustainable energy production emphasizes the importance of the conversion of raw starch to biofuels. However, the production of bioethanol/biobutanol/biohydrogen from starchy material itself is an energy-intensive process, and therefore, any small contribution to reducing energy consumption would be highly desirable. RSDE have been proven to be fruitful in this regard by bypassing the energy-intensive starch gelatinization step. Unfortunately, this has not been applied on an industrial scale due to various limitations. Thus, continuous efforts

are being made to obtain industrially efficient RSDE which can directly act on various raw starches. However, the increasing demand for food crops to feed the ever-increasing population has brought some impediments in its use for biofuel production. Thus, the current trend is to seek for underutilized crops or starchy agricultural wastes as feedstock for biofuel production. Furthermore, the cost-effective bioconversion of raw starch into bioethanol/biobutanol/biohydrogen requires consolidated bioprocessing; several attempts of which have already been made. However, the major challenges are the production of enzymes with higher specific activities, which can convert raw starch into glucose at a faster pace (Zyl et al. 2012). Sometimes biofuel production is also affected by end-product inhibition such as sugars and oligosaccharides, as the enzymes lose their activity or not stable at these conditions (Visser et al. 2015). Thus, the focus is also placed on the development of more efficient and cost-effective enzymes. However, these goals are not simple and require concerted research efforts (Zyl et al. 2012).

Recent trends of biofuels production in one pot (one-step production, consolidated process) seems to be one of the most promising approaches. Strategies involved here are either the heterologous expression of RSDEs directly on the cell surface or secreted directly into the fermentation broth by the fermenting organism to enable simultaneous hydrolysis, liquefaction, and fermentation (Cinelli et al. 2015). However, little success has been achieved in developing an industrial recombinant strain for direct ethanol/butanol production from raw starch (Cinelli et al. 2015). Though some native strains of *Saccharomycopsis fibuligera*, *Schwanniomyces castelli,* and *Saccharomyces diasticus* have been reported to produce both amylases and ethanol (Cinelli et al. 2015), and even some strains belonging to *Clostridium* sp. are capable of fermenting starchy substrates (Ranjan and Moholkar 2012), but none of them reached up to industrial scale (Cinelli et al. 2015). Therefore, further research should focus on engineering the existing fermenting strains for consolidated bioprocessing of raw starch into biofuels. Search for new, efficient raw starch digesting microorganisms is an alternative. Further, scientists are also looking for organisms which contain more than 50% of their biomass in the form of starch or glycogen, such as microalgae belonging to the genera *Chlorella, Dunaliela, Chlamydomonas, Scenedesmus,* and *Spirulina* as non-food competitive biomass for biofuel production (Cinelli et al. 2015). However, methods for harvesting microalgal cells are challenging and non-viable (Zyl et al. 2012). Attention is also devoted to exploring possibilities of plant genetic modification to increase the endogenous amylase level. Syngenta, a biotech company had developed a corn variety, expressing the α-amylase gene with an improved pH and temperature profile, thus reducing the energy-intensive starch liquefaction. Successful metabolic engineering of fermenting strains could also allow redirection of glucose transformation to ethanol/butanol, to several alternative products. This includes lactic acid (Sauer et al. 2010), artemisinin (Xu et al. 2008), glutathione (Yoshida et al. 2011), etc. This would add value to the current raw starch utilization process. Ledesma-Amaro et al. (2015) engineered *Yarrowia lipolytica* to overexpress α-amylase from *Oryza sativa* and glucoamylase from *A. niger,* so that the modified strain could produce a high amount of lipids using starch as a sole carbon source. Thus, there are

numerous new avenues to explore, including raw starch conversion into lipids as biodiesel precursors. Although substantial work has already been done on RSDE, the exploration of enzymes to produce bioethanol, biobutanol, biohydrogen, and other value-added products at the industrial scale is still at the nascent stage. This offers a great avenue for the exploration of novel RSDE which could directly find its application in the above-mentioned industries.

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Bamboo Valorization by Fermentation and Enzyme Treatment

4

Divyajyoti Biswal, Saurabh Shinkhede, and Sachin A. Mandavgane

Abstract

Bamboo is found in abundance in India and known to be the fastest growing perennial grass. It is a tough and robust crop which can thrive under limited water and/or chemical fertilizers and pesticides. Previously considered as forest produce, bamboo has recently been declassified as a grass variety, meaning retracted restrictions on its cultivation, harvest, and transport. This ordinance, recently passed by the Government of India, aims at projecting bamboo as one of the financially beneficial plants. The main objective is to fuel the growth of rural agro-business start-ups in order to bring financial independence as well as elevated living standards among the rural mass. From an environmental perspective, bamboo stands out as an ideal choice for several applications ranging from housing and construction to alternate fuel production. This chapter discusses the several potential applications bamboo can have in several sectors and enzymatic extraction of fibers from mechanically and chemically treated bamboo. Bamboo can be used for the generation of bioethanol. Bamboo shoots are a delicacy in most south Asian countries. Four enzymes have been suggested in the text, i.e., Lyase, Cellulase, Xylanasse, and Laccase, which can be used in optimized quantity to extract the lignocellulosic fibers of mechanochemically treated bamboo.

Keyword

Bamboo · Fermentation · Saccharification · Ethanol · Enzymatic hydrolysis

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4.1 Introduction

Bamboo is a woody perennial grass that is found in the tropical and subtropical evergreen and evanescent forest formations of Asia-Pacific. India, China, and Myanmar are major producers for bamboo contributing to around 80% of the bamboo production around the world. India has the global bamboo growing landscape with 136 (125 indigenous and 11 exotic) species in its credit, 23 genera which is spread over 13.96 million ha (ISFR 2017); and thus is the second largest bamboo growing country in the world (FSI 2011). However, India's share in global bamboo market is only 4%, but it possesses 45% of global bamboo growth (Kumar et al. 2014) (Fig. 4.1).

Bamboo plays a significant role in daily human lives. It is used from pickle preparation (Yeasmin et al. 2015) along with edible products and traditional Ayurvedic medicine to construction materials along with edible products and traditional medicine to construction materials. It is also a vital part of paper and pulp industry. Strong antioxidant activity is shown by solvent extraction of *P. bambusoideae* and *P. pubescens* (Mu et al. 2001). It is also found that cyanogenic glycosides are present in the mature bamboo leaves (Das and Chakraborty 2008). Also, high-quality charcoal can be manufactured from the adult bamboo culm. Moreover, it is used for the construction of several musical instruments. It has multitudinous application in the construction of house as stated earlier such as erecting pillars, floors, doors and windows, room separator, and rafters (Das and Chakraborty 2008). It is also employed for the construction of guard wall/dam for water bodies and river bank. Bamboo is also efficacious for impeding soil erosion and conserving soil moisture (Kleinhenz and Midmore 2001; Mailly et al. 1997).

Interestingly, bamboo shoots as well as culms find an application in production of bioethanol via fermentation. The chemical composition of the bamboo cell walls consists of cellulose, hemicelluloses, and lignin. Conversion of such lignocellulosic biomass into ethanol facilitates a sustainable pathway to energy production, thus using bamboo as a feedstock for the production of bioethanol post dilute acid pretreatment and enzymatic saccharification, among others. The process of lignocellulosic biomass into bio-ethanol includes pretreatment, hydrolysis, fermentation, and distillation. Pretreatment is the most vital step in the conversion of cellulose to ethanol since it removes hemicelluloses and lignin, thereby increasing the porosity of materials which aids in improvement of enzymatic saccharification (Hendriks and Zeeman 2009). Cheaper bamboo feedstocks make the bioethanol production more economical as the price of the feedstock contributes to 50% to the production cost.

Moreover, enzymatic digestion of bamboo in fiber extraction is also gaining momentum as research progresses. Natural bamboo fibers exhibit rising demands in textile industry due to its serviceable properties, renewability, and abundance as a resource. However, deficient quality aspect of bamboo fibers limits their usage in the textile industry. Enzymatic treatments on mechanochemically modified bamboo can prove to be an efficient way to extract non-cellulosic finer natural bamboo fibers which will be discussed further.

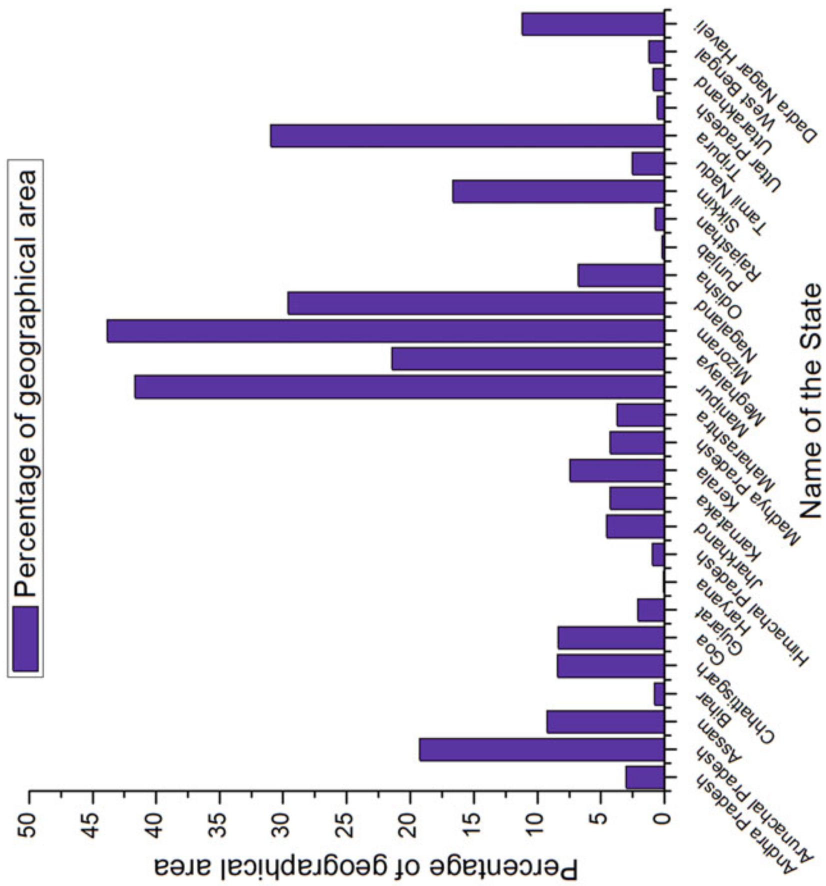

Fig. 4.1 The state-wise distribution of bamboo resources in India. (Source: FSI 2011)

With the myriad range of applications, bamboo can prove to be a useful bioresource for systematic and integrated chemical recovery process design.

4.2 Valuable Products from Bamboo by Fermentation Route

The textbook definition of fermentation describes it as a process involving the breakdown of a chemical or a compound by bacteria, yeast, or other microorganisms. Fermentation is used for the production of many value-added chemicals like ethanol, lactic acid, butanol, acetone, etc. from biomass feedstock. The choices of microorganism and substrate are crucial to deciding the final product. Moreover, the process parameters like temperature, pH, substrate concentration, aeration, and agitation decide the optimal production conditions. The fermentation route of production stands on safer grounds when environmental prospects are concerned. With increasing research and demand for alternate fuel, bioethanol appears to be one of the prime contenders in the race as it qualifies in accordance with energy as well as environmental standards/respects/regards. Shapouri et al. (1995) have reported that the energy content of ethanol is greater than what is required for its production. Production of bioethanol using different biomass feedstock and microbial strain has been reported by scientists. The details of different strains and biomass feedstock used in bioethanol fermentation are mentioned in Tables 4.1 and 4.2, respectively.

4.2.1 Bamboo Culms as Fermentation Feedstock

Often bamboo is regarded as a weed in forestry, a plant that suppresses the growth of other trees around because of its exceptional growth rate. Its widespread distribution across Asia has triggered its use for a number of beneficial purposes, like a source of fiber (Zhang et al. 2007; Scurlock et al. 2000), regional cuisine (bamboo shoot is one of the many south Asian delicacies), a potential substrate for the production of fuel ethanol (Zhang et al. 2007), biogas (Kobayashi et al. 2004), and other valuable products. With the increasing rate of consumption of conventional fossil fuels, the rate of their depletion is soaring. The present-day situation calls for an appropriate alternative to these conventional fossil fuels. Bioethanol appears to be one of the prime contenders in the race, owing to its satisfactory energy and environmental regard (Farrell et al. 2006). In India, around 125 indigenous and 11 exotic species of bamboo are reportedly found. A majority of the bamboo produced in the country belong to the northeastern states followed by Chhattisgarh, Madhya Pradesh, and the Western Ghats. Among the islands, Andaman and Nicobar hosts a significant fraction of the grass (ISFR 2017).

The most crucial step during the production of ethanol from bamboo is cellulose hydrolysis. Since the major constitution of bamboo is lignocelluloses, hydrolysis step plays a vital role in releasing the sugars embedded in the complex polysaccharide network. Several alternatives to the hydrolysis of lignocellulosic biomass are

Table 4.1 Bacterial species which produce ethanol as main fermentation product

Bacterial species	Pathogenicity	Millimol of ethanol produced per millimol of glucose metabolized	References
Clostridium sporogenes	Non-pathogenic	Up to 4.15	Miyamoto (1997)
Clostridium indoli	Pathogenic	1.96	
Clostridium sphenoides	Non-pathogenic	1.8	
Clostridium sordelli	Pathogenic	1.7	
Zymomonas mobilis	Non-pathogenic	1.9	
Zymomonas mobilis subsp. *Pomaceas*	Non-pathogenic	1.7	
Spirochaeta aurantia	Non-pathogenic	1.5	
Spirochaeta stenostrepta	Non-pathogenic	0.84	
Spirochaeta litoralis	Non-pathogenic	1.1	
Erwinia amylovora	Non-pathogenic	1.2	
Escherichia coli KO11	Non-pathogenic	0.7–0.1	Dien et al. (2003), Matthew et al. (2005)
Escherichia coli LY01	Non-pathogenic	40–50 g/L	Dien et al. (2003)
Leuconostoc mesenteroides	Non-pathogenic	1.1	Miyamoto (1997)
Streptococcus lactis	Non-pathogenic	1.0	
Klebsiella oxytoca	Non-pathogenic	0.94–0.98	Matthew et al. (2005)
Klebsiella aerogenes	Non-pathogenic	24 g/L	Ingram et al. (1998)
Mucor sp. M105	Non-pathogenic	–	
Fusarium sp. F5	Non-pathogenic	–	

reported, like concentrated acid hydrolysis (Sun et al. 2011), dilute acid hydrolysis (Goldstein and Easter 1992), enzymatic hydrolysis, microwave-assisted hydrolysis (Wang et al. 2015), etc. However, dilute acid hydrolysis of bamboo feedstock at elevated temperatures and high pressure results in the formation of toxic compounds that hinder fermentation and limits the bioethanol yield within 50–60%. Sun et al. (2011) have reported the use of concentrated sulfuric acid for the hydrolysis of bamboo feedstock and subsequent continuous ethanol fermentation. Lower

Table 4.2 Various biomass feedstocks used for the production of bioethanol

Sl. No.	Feedstock	Reference
1	Microalgal biomass	Harun et al. (2010)
2	Corn stover	Kadam and Mcmillan (2003)
3	Barley	Gibreel et al. (2009)
4	Potato peel	Khawla et al. (2014)
5	Sweet potato	Srichuwong et al. (2011)
6	Jackfruit	Chongkhong et al. (2012)
7	Sweet sorghum	Almodares and Hadi (2009)
8	Rice straw	Hyoun et al. (2014)
9	Wheat straw	Hyoun et al. (2014)
10	Sugarcane bagasse	Dias et al. (2012)
11	Agro waste	Sarkar et al. (2012)
12	Yeast	Hyoun et al. (2014)

condensation and recovery costs coupled with reusability of sulfuric acid are what make this process economically viable (Goldstein and Easter 1992). Goldstein et al. (1989) have reported the recovery of sulfuric acid by electrodialysis and pointed out that the crucial parameter in determining the release of sugar from the complex polysaccharide network is the ratio of acid to substrate (AR). Optimization studies by Clausen and Gaddy (1993) have indicated that a two-stage hydrolysis process with an AR of 7, i.e., 90%, is almost equivalent in terms of yield to that from a single-stage hydrolysis process with and AR of 35. Iranmahboob et al. (2002) further reduced the AR from 7 to 2 in a two-stage hydrolysis setup and achieved a glucose recovery efficiency of 78–82% which is beneficial, as the acid recovery cost is significantly reduced without affecting sugar concentration by a significant margin. Sun et al. (2011) in their study to demonstrate the effect of concentrated acid hydrolysis on sugar recovery and its subsequent continuous ethanol fermentation have used a special reactor system for hydrolysis using concentrated H_2SO_4 in which the reaction vessel fitted with a helical propeller blade remains dipped in the oil bath of the reactor. The economic viability of H_2SO_4 is attributed to its easy recovery and reusability.

4.2.1.1 Bamboo Saccharification Using Concentrated H_2SO_4

Saccharification is a two-step process: solubilization followed by hydrolysis. Solubilization was carried out as overnight oven dried (60 °C) bamboo chips of dimension 1–3.75 mm were mixed gently with 75 wt% of H_2SO_4 at various ARs from 1 to 2 at 50 °C and 150 rpm for 30 min. This resulted in the formation of a paste which marks the end of solubilization step. Water at 90 °C was added to the mix to generate a suspension of 27 wt% acid and was stirred at 85 °C for an hour, marking the end of the hydrolysis step. The slurry was then pressed at 20 MPa for 20 min to drain out the hydrolysate which contained the released oligosaccharides which was further diluted ten times to be hydrolyzed in batch mode at a pH of 0.8 and temperature 120 °C for 30 min. The slurry was pressed to remove the solid from hydrolysate, which contained the released simple sugars that was diluted ten times and hydrolyzed in

batches. The effect of temperature on the release of oligosaccharides from the complex carbohydrate network was also determined and efficiency of release was calculated by the following formula (Sun et al. 2011):

$$\text{Sugar recovery efficiency}(\%) = \frac{\text{Total amount of glucose and xylose in saccharified liquid}}{\text{Total amount of glucose and xylose in bamboo chips}} \times 100$$

4.2.1.2 Continuous Ethanol Fermentation

A specialized tower type reactor was used for the purpose fitted with a solid–liquid–gas separation unit at the upper section. Continuity of air supply was maintained from the bottom of the reactor via a ball filter. Peristaltic pumps controlled the addition of unsterilized hydrolysate and other essential nutrient chemical solutions like ammonium and magnesium sulfates, potassium dihydrogen phosphate, etc. in appropriate amounts, with constant monitoring of the glucose and xylose concentrations on a daily basis. Constitution of bamboo was determined using standard analytical methods which identified (Sun et al. 2011) and quantified constitutions like lignin, pectic residue, oligosaccharides, proteins, etc.

4.2.1.3 Effect of Temperature and Acid Ratio on Sugar Concentration

With increasing acid ratio, the concentration of glucose and xylose seem to reduce when analyzed prior to hydrolysis. However, it was also observed that oligosaccharide hydrolysis significantly boosts up the sugar recovery efficiency (Fig. 4.2). The analysis done to determine the effect of temperature showed that 80 °C has the optimal sugar recovery efficiency. The trend is increasing up to the temperature of 80 °C and reduces thereafter. Figure 4.2 represents the trends as explained by Sun et al. (2011).

4.2.1.4 Catalyzed Steaming

Tsuda et al. (1998) reported the effect of catalyzed steaming as a pretreatment procedure for enzymatic hydrolysis of bamboo. Commonly, steaming is regarded as an effective pretreatment procedure for decomposition of lignocellulosic material into its constituents. In this method, hemicelluloses become water soluble, and susceptibility of cellulose to enzymatic degradation is increased (Tsuda et al. 1998). In this study, the bamboo culms were air dried and crushed using hammer mill. Screening was done to remove any particle of size less than 1 mm. Prior to the enzyme treatment, the culms were sprayed with acid catalyst and refrigerated for a few days. The catalysts used for the purpose were $AlCl_3$, $Al_2(SO_4)_3$, $FeCl_3$, NH_4Cl, $(NH_4)_2SO_4$, acetic acid, maleic anhydride, succinic anhydride, and phthalic anhydride. The extracted culms were then subjected to steam treatment at 197 °C followed by air drying. Portions of steamed and ground solid were subjected to hot-water extraction and enzyme hydrolysis was carried out using cellulose (Sudo et al. 1976). The hydrolysis was done in 0.1 M sodium acetate buffer for 40–48 h,

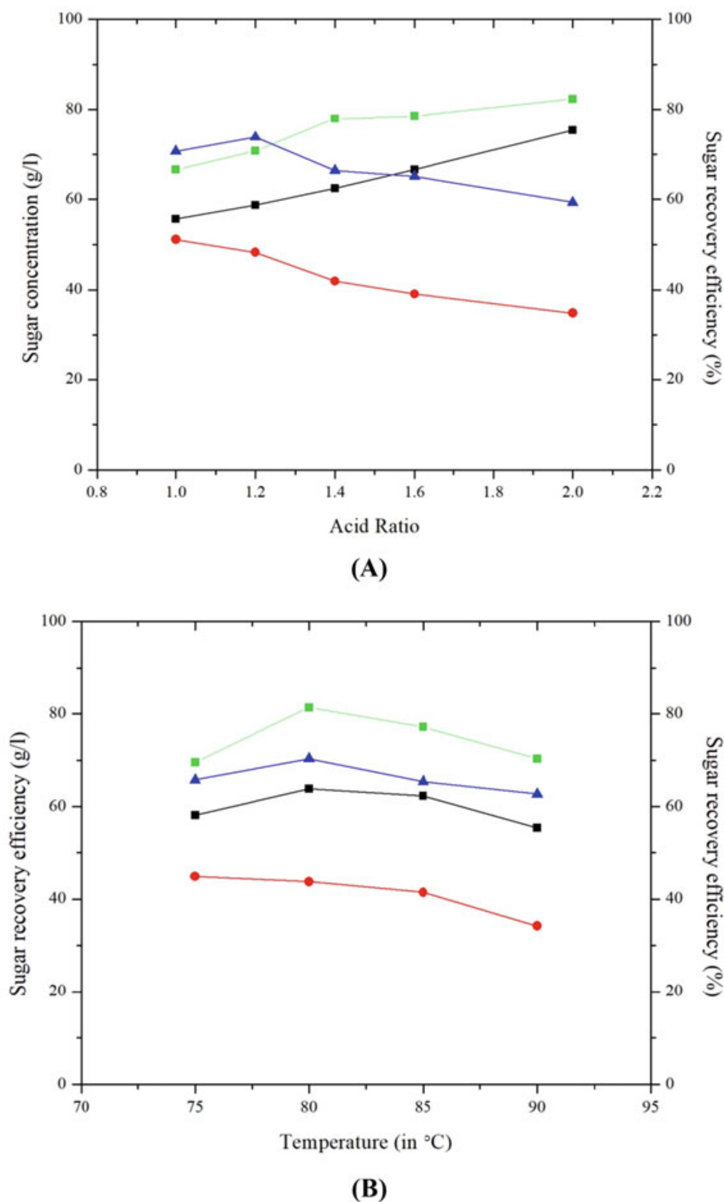

Fig. 4.2 (**a**) Effect of AR and (**b**) effect of temperature on sugar recovery efficiency. *Note*: Blue triangle, glucose concentration, red circle, xylose concentration; black square, sugar recovery efficiency (SRE) before hydrolysis; green square, SRE after hydrolysis. (Adapted from Sun et al. (2011) and redrawn)

and the amount of reducing sugars formed was analyzed (Michael 1951). Extraction using $AlCl_3$, $Al_2(SO_4)_3$, $FeCl_3$, NH_4Cl, $(NH_4)_2SO_4$ showed exceptional percentage enzyme hydrolysis after stream treatment while the yield of reducing sugar was the maximum using $AlCl_3$ (5 mmol). An increment of 2% was observed in terms of sugar yield when catalytic steam treatment was used prior to enzyme hydrolysis indicating better cleavage of the complex polysaccharide network (Tsuda et al. 1998).

4.2.2 Bamboo Shoot as Fermentation Feedstock

Bamboo shoot refers to the young edible bamboo plants that have just emerged from the ground. Typically around 20–30 cm long and about one pound in weight, these are a delicacy in most of the South Asian countries. In India, parts of the northeast consume bamboo shoots as a primary food item. The freshly harvested bamboo shoot appears pale yellow in color and tastes sweet if consumed immediately. However, all the shoot varieties are not edible. Their color, length, taste, and other physicochemical properties are affected largely by the climate, pH of soil, fertility parameters, air moisture, etc. Bamboo shoots are seasonal and perishable, yet rich in nutritional aspects. Studies suggest that bamboo shoots are entirely devoid of fats and cholesterol, whereas they are rich in carbohydrates and dietary fibers, with the moisture content of around 88%. Bamboo shoots contain around 17 different types of essential amino acids and are home to several essential mineral elements, namely Co, Cr, Zn, Mn, Mg, Ni, Cu, etc. Apart from these, the shoots contain several vitamins and antioxidants like phenols, flavones, steroids, etc. Several preparations of these shoots are used for several purposes, like the boiled shoots are served as appetizers, whereas the decoctions of the shoots are used for wound treatment (as it possesses antimicrobial properties as well). One of the common methods used to make the shoots more delicious and appealing is fermentation. Dried-fermented, fermented-canned, and fermented-sliced bamboo shoot preparations are most common in traditional bamboo shoot cookeries in the South Asian countries (Choudhury et al. 2012). Several different preparation methods may be listed involving several mix and combination of spices and ingredients, like the Indonesian thick coconut milk and spices recipe, the Indian *ushoi* in Manipur, *Apa Tanis* in Arunachal Pradesh (Choudhury et al. 2012), and so on.

4.2.2.1 Simultaneous Saccharification and Fermentation

Shimakowa et al., have studied the consequences of growth stage on enzymatic saccharification and simultaneous saccharification and fermentation of feedstock (bamboo shoots) for production of bioethanol. The culms and shoot of *P. bambusoides* were cut and dried at a temperature of 45 °C until the moisture content was down to <10% followed by pulverization in a rotor mill fitted with a sieve ring of 0.5 mm. The enzyme preparations used were Meicellase (Manufacturer—Meiji Seika Co., Ltd., Source organism—*T. viride*, 332 FPU g_1; b-glucosidase activity, 1050 U g_1) and Cellulosin TP 25 (Manufacturer—HBI

Co., Ltd., Source organism—*T. viride*, 157 FPU g_1; b-glucosidase activity, 344 U g_1; xylanase activity, 17,200 U g_1). The enzyme hydrolysis was performed by formulating a preparation of 20 mg of milled sample with enzyme solutions in 1 mL of 50 mM citrate buffer, pH 4.8. The mixture was then incubated at 40 °C with simultaneous shaking. The reaction was stopped at 100 °C for a few minutes following which the amount of saccharification was calculated (Shimokawa et al. 2009).

Saccharomyces cerevisiae NBRC 2347 was used for the purpose. Precultured yeast was pelletized by centrifugation and washed with deionized autoclaved water. The pellet was then added to SSF experiment flasks, net volume 50 mL. The following are the flask contents:

- 5% milled bamboo
- 25 mM Na-citrate buffer (pH 4.8)
- Enzyme preparation.
- 0.25 g (wet weight) of the washed yeast pellet.

The reaction mixture was stirred at 30 °C. the experiments were carried out in triplicates (Shimokawa et al. 2009).

4.2.2.2 Effect of Growth Stage on Enzyme Hydrolysis

The results show initial awry lignin distribution in the shoot fractions taken from different plant heights above the ground. The distribution evened out after the branch spread, prior to which it was greater at the bottom and lesser at the top. Table 4.3 summarizes the lignin yield and saccharification (Shimokawa et al. 2009).

4.2.2.3 Ethanol Yields

Harvesting of shoots from *P. bambusoides* and *P. pubescens* before branch spread was carried out for SSF experiments. For *P. bambusoides*, the yield of ethanol was found to be 123 g kg^{-1} after 7 days of fermentation with 2 FPU g^{-1}, roughly 71% of the theoretical yield (hexose conversion based). The theoretical ethanol yield as per hexose conversion of *P. bambusoides* and *P. pubescens* were determined to be 173.44 and 171.83 g kg^{-1}, respectively. The maximum ethanol yield of 169 g kg^{-1} could be achieved after 7 days of SSF (with 12 FPU g^{-1}); 139 g kg^{-1}

Table 4.3 Acid insoluble lignin percentage for *P. bambusoides*

Height from ground (m)	Acid insoluble lignin percentage					
	0–1	1–2	2–3	3–4	4–5	5–6
1	2.9					
2	4.0	2.4				
3	9.0	5.9	4.7			
4	13.4	6.4	3.7	5.2		
5	13.3	10.8	9.3	6.7	4.9	
7	17.2	16.3	16.3	15.5	15.0	15.9

(81%) ethanol was also produced from *P. pubescens* after 7 days of SSF, although the yields were somewhat lower than those of *P. bambusoides*.

4.3 Enzymatic Extraction of Bamboo Fibers

The main constituents of bamboo culms are holocellulose (60–70%), pentosans (20–25%), hemicelluloses and lignin (each amounted to about 20–30%), and minor constituents like resins, tannins, waxes, and inorganic salts. Four enzymes, pectinlyase, xylanase, laccase, and cellulase, can be used to remove pectin, hemicellulose, and lignin and to loosen the compact structure of fibers, respectively (Liu et al. 2012). Cellulose is the primary content of the bamboo fiber linked together with exceptionally strong lignin bonds. It is described as a high molecular weight carbohydrate polymer with monomeric units of β-1,4-linked anhydro-D-glucose. Cellulose chains are biosynthesized and clustered into crystalline domains called microfibrils and amorphous regions. Bamboo contains about 40–55% alpha cellulose (Li 2004). Significant cellulose content in the bamboo fiber can be exploited for two main purposes, i.e., energy production and sustainable textile manufacturing. In this section, we shall discuss about various enzymes that can be used for the extraction of bamboo fiber. Before jumping to the enzyme treatment, we shall discuss the existing methods for the extraction of bamboo fiber.

Washing of bamboo strips was carried out by distilled water under ambient conditions of temperature and pressure. This step is critical in the removal of dust and impurities off the surface of the strip followed by a gentle rolling for BF extraction. Cellulosic fiber removal becomes essential in order to be able to tap into the lignin and hemicellulosic material from the BFs. This was achieved by:

- Alkali treatment: To dissolve the pectins and the hemicelluloses followed by removal of dissolved components by thorough washing with deionized water.
- Bleaching: Removal of phenolic compounds/pigments in lignin causes the BFs to whiten, 5 wt% sodium hypochlorite solution (NaOCl), at 30 °C, for 3 h being used for the purpose

Deshpande et al. (2000) have studied chemical treatment of de-lignification where lignin is dissolved in NaOH solution and then cellulosic fibers are extracted very easily. This is followed by the mechanical treatment using compression molding technique (CMT) and rolling mill technique (RMT).

Conventionally, chemical and mechanical treatments can be employed to extract the fiber. But these methods affect the fiber quality as they use harmful chemicals and also reduce its mechanical strength (Liu et al. 2012). Kozlowski et al. (2006) mentioned in his study that for ramie and sunn hemp fibers, the optimal process modification can be achieved by combining three different methods, i.e., initial mechanical processing, mild chemical treatment, followed by enzymatic treatment. Hence, a similar enzyme treatment is proposed to extract non-cellulosic materials from bamboo fibers, so that it can be used in textile industry extensively.

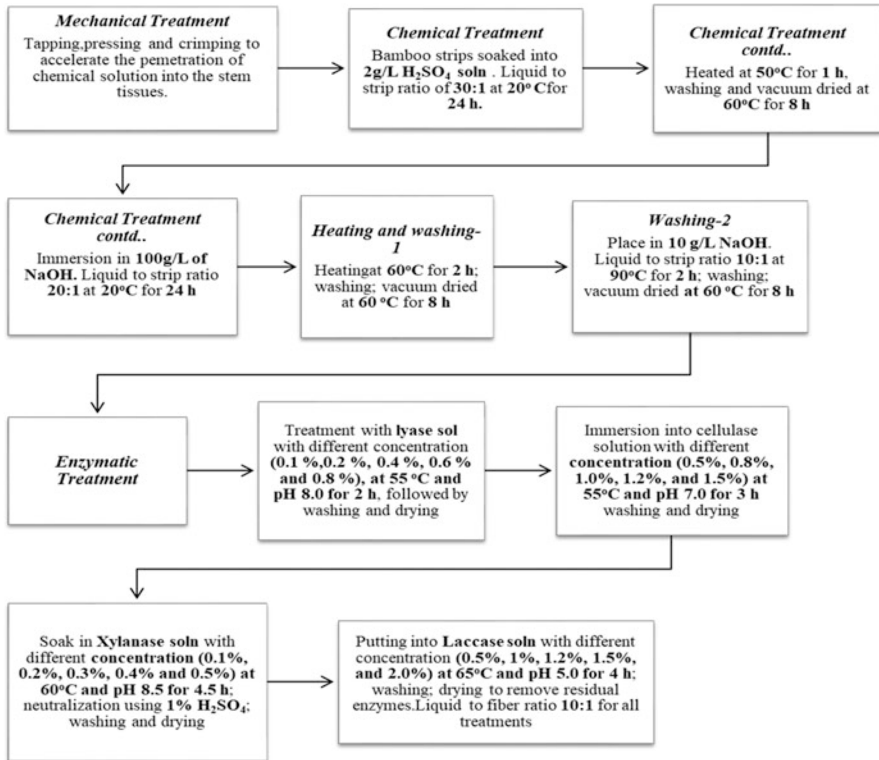

Fig. 4.3 The mechano-chemical treatment followed by suggested enzyme treatment (Liu et al. 2012)

The bamboo fibers are treated mechano-chemically to reduce the strength of lignin bonds to be further broken easily using four different enzymes. Liu et al. have studied enzymatic treatment using four different enzymes, i.e., xylanase, pectinlyase, cellulase, and laccase. These are used to slacken the compact structure of fibers and remove pectin, hemicellulose, and lignin, respectively, in an optimized concentration (Liu et al. 2012) (Fig. 4.3).

4.4 Conclusion

The recent notification by Ministry of Environment and Forest to declare bamboo as grass can achieve two national priorities, namely increasing the income of farmers and increasing the green cover of the country. This has created enabling environment for the promotion of bamboo production (even outside forest), setting up of units to make value-added products from bamboo and linkage with market. A new agri business opportunity based on bamboo feedstock has been opened up which can

strengthen rural economy. The application of fermentation and enzymatic extraction process to bamboo feedstock surely has great potential for value-added product generation. The relatively simple and cost-effective processing strategy of fermentation should enable rural small-scale enterprises to thrive and flourish. However, there is a need of process development and demonstration of making of bamboo-based products which have market demand and even serve as import substitute. This chapter effectively describes the use of bamboo feedstock as potential sources for alternate fuel as well as food and fabric. India imports bamboo fibers and silica which has a huge domestic market demand. Indigenous production of the same can be financially beneficial to the farmer as well as the country.

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5 Recent Developments in Pretreatment and Enzymatic Hydrolysis for Cellulosic Bioethanol Production

Puneet Kumar Singh, Snehasish Mishra, Sanjay Kumar Ojha, and Kalyani Naik

Abstract

Second-generation biofuels like bioethanol from lignocelluloses instead of sugar or starch have demonstrated environmental and economic benefits. Bioethanol is conventionally produced from sugar through a two-step saccharification and fermentation, primarily by yeasts (like *Saccharomyces cerevisiae*) and bacteria (like *Zymomonas mobilis*, *Escherichia coli* and *Klebsiella oxytoca*) either separately or simultaneously. Pretreatment of the substrates reduces the size, breaks down the fibres, swells and softens the biomass, reduces the crystallinity, solubilises hemicellulose and/or removes lignin or has a combined effect of any of these, with a major objective to enhance the available surface area for enzymatic hydrolysis. Pretreatments could be physical (microwave and steam explosion, ultrasound, mechanical and extrusion), chemical (acids, alkalis, ozone, organosolvs and ionic liquids) and biological (cellulases, hemicellulases and other accessory enzyme-based measures). Physicochemical pretreatments and combined physical and chemical measures involve ammonia fibre explosion, ammonia recycle percolation, wet oxidation, CO_2 explosion, etc. A combination of physicochemical followed by biological pretreatment (hydrolysis by cellulases, hemicellulases and accessory enzymes) is reportedly the most effective strategy for high sugar yields that would further facilitate the net bioethanol conversion potential. This write-up is an effort to put in perspective the technological breakthroughs and recent advancements in bioethanol production for energy self-sufficiency from sustainable/renewable sources.

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Keywords

Cellulose · Ethanol · Hemicellulose · Hydrolysis · Lignocellulose · Pretreatment

5.1 Introduction

Lignocellulose is largely composed of three major macromolecules, hemicellulose, cellulose and lignin. It is one of the most recalcitrant forms of biological materials in nature. The two main components that make it recalcitrant are cellulose (a polymeric structure of glucose molecules) and lignin (a polymeric structure of lignols). Hemicelluloses consist of heterogeneous structures including galactose, xylose, mannose, arabinose and rhamnose. It is necessary to hydrolyse the complex lignocellulosic structure to fermentable sugar moiety before the microorganisms convert it to ethanol. Hydrolysis is an economical way of biochemical conversion of lignocellulose to produce ethanol. Various physicochemical and biological pretreatments are employed to ensure this, overcoming the refractory nature of the complex lignocellulosic biomass. The developing and evaluating combined pretreatment approaches with reduced dependency on enzyme hydrolysis and reduced bioconversion time. The precipitated lignin portion resulting from such processes is removed by filtration from the sugar solution during the saccharification process (Alvira et al. 2010). The write-up that follows discusses various pretreatment strategies, biochemical (enzymatic) hydrolysis followed by microbial interventions for enhanced bioethanol production.

With the growing demand for energy in general and renewable energy in particular, there has been a global rise in the various research and development interventions to enhance energy sourcing both qualitatively and quantitatively. The versatility in the use of bioethanol in the same context puts it ahead of many of its renewable counterparts. Though a combined strategy would enhance the yield and productivity as is construed from available literature, there seem to be technological gaps that are yet to bridge. In light of this, the chapter is an attempt to put the developed technologies in perspective, developing technology in context and the futuristic technological interventions in prospective.

5.2 Pretreatment Methods for Lignocellulosic Biomass

Owing to the wide variations in composition and bonding patterns in the numerous forms of lignocelluloses in nature, the pretreatment strategies also vary greatly. Various pretreatment measures that have been established since long could be categories as physical, chemical and biological (Fig. 5.1). However, combinations of these treatments are often employed to achieve the desired objective.

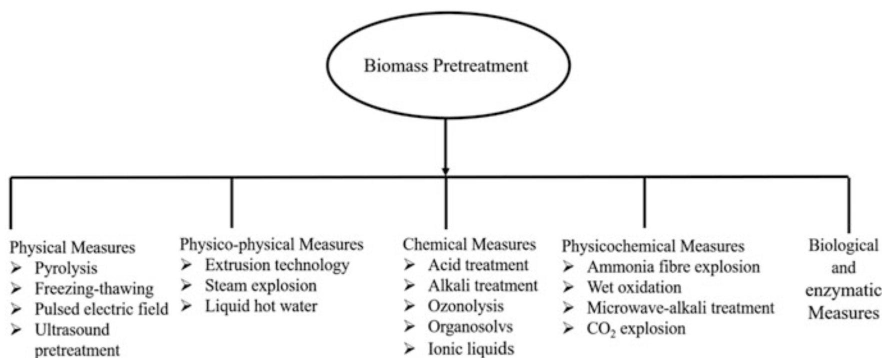

Fig. 5.1 Biomass pretreatment measures applied for lignocellulose degradation and enzyme hydrolysis for saccharification

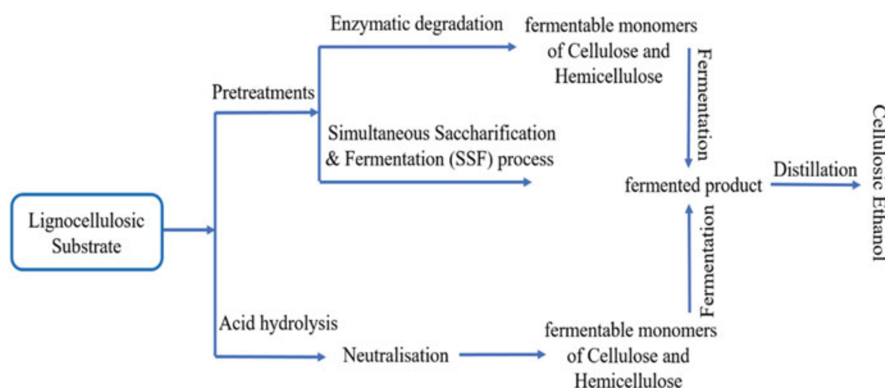

Fig. 5.2 Flowchart of SSF and SHF for the generation of cellulosic ethanol

5.2.1 Physical Measures

Lignocellulose is covered around by lignin and hemicelluloses and presents an external and an internal surface. The internal one is dictated by the porous structure of cellulose microfibrils, whereas the external one is by the particle size and the overall shape of biomass. The very first step in degradation of lignocellulose is subjecting it to physical/mechanical means. The main objective of this step is to reduce the size and crystalline nature of this recalcitrant biomass, thereby enhancing its chemical and microbial accessibility. Crystallinity of cellulose and the percent hemicellulose and lignin contents are factors that primarily influence the pretreatment strategies. Various pretreatment methods are engaged to remove and/or degrade lignin and hemicellulose, thereby exposing the cellulose for maximum hydrolysis for ethanol production. Similarly, the rate of hydrolysis of cellulose microfibrils depends on its amorphous and crystalline nature (Fig. 5.2).

Physical treatments can be classified as thermal (viz., pyrolysis, microwaving, freezing-thawing), hydrothermal, electrical, chipping, mincing, grinding, sieving, extruding, etc. (Maurya et al. 2015; Saini et al. 2015). Many milling methods (viz., roll-ball, colloidal, hammer milling) prior to subjecting the substrate to enzymatic decomposition enhance its sugar convertibility.

5.2.1.1 Pyrolysis

It involves degrading the biomass at high temperature in absence of oxygen; it degrades cellulose to H_2, CO and residual char during the process. The process involves an initial high temperature to release volatile compounds, condensing these volatile compounds and continuing with secondary autocatalysis. The process results in the recovery of glucose-rich liquid fraction. The quality and quantity of the recovery depend on the biomass properties (such as composition of lignocellulose, particle size and pore size) and several process parameters (such as rate of heating, temperature and reaction time) (Canilha et al. 2012).

5.2.1.2 Freezing-Thawing

Freezing-thawing is a relatively new approach for biomass pretreatment. Freezing at $-20\ ^{\circ}C$ and thawing at $37\ ^{\circ}C$ dehydrate the biomass that results in cell wall damage, thereby creating larger accessible surface area for microbial activity. Mood et al. (2013) and Smichi et al. (2016) employed this process for enhanced enzyme hydrolysis of rice straw and sea rush (*Juncus maritimus*). However, the very high energy requirement for the process makes it less attractive.

5.2.1.3 Pulsed Electric Field

This treatment involves applying short burst high voltage electric supply to biomass placed between the two electrodes. High-voltage electric field damages the biomass by changing the membrane structure. Membrane damage creates pores on the biomass surface and improves its microbial accessibility for hydrolysis, thereby resulting in high sugar yields described by Peral (2016).

5.2.1.4 Ultrasound Pretreatment

In this, the substrate is subjected to sound waves of high frequency. Ultrasound creates cavities in the substrate as a result of which enzymes easily transport through and hydrolysis is enhanced. Alvira et al. (2010) reported ultrasound pretreatment of lignocellulose to extract hemicellulose, cellulose and lignin from it. Yachmenev et al. (2009) found maximal cavity formation when the substrate was ultrasonically treated at $50\ ^{\circ}C$, the temperature that is also suitable for enzymatic activity.

However, not all the mechanical means are that popular owing to the additional costs (including the energy costs) involved in the process. For instance, high power consumption during grinding, milling, prilling and extruding makes them economically unfeasible, thereby being not-so-popular by Maurya et al. (2015).

5.2.2 Physico-Physical Measures

Instead of subjecting the substrate to a single physical pretreatment, it is often recommended to consider multiple physical means either in tandem or together which leads to the desired level of physical and chemical alterations in the lignocellulose.

5.2.2.1 Extrusion Technology

Extrusion technology, as propounded by Karunanithy and co-workers (Karunanithy et al. 2013; Karunanithy and Muthukumarappan 2010), is a combined physico-physical treatment of heating, mixing and cutting followed by enzymatic/biological treatment that transforms lignocellulose to sugars. This method converts lignocellulosic biomass to small-sized fibres and increases the availability of carbohydrate molecules for enzymatic degradation.

5.2.2.2 Steam Explosion

It is a hydrothermal treatment during which the substrate is exposed to high vapour pressure for few seconds to minutes, and then the pressure is released. Autohydrolysis occurs once high temperature is achieved, thereby enhancing the conversion of acetyl groups to acetic acid. Additionally, water acts as an acidifier at very high temperatures. When the pressure is suddenly reduced, the fibres are separated due to sudden decompression mentioned by Alvira et al. (2010). The steam explosion results in redistribution within the substrate and, to some extent, the release of lignin along with partial hydrolysis and solubilisation of hemicellulose described by Sun et al. (2016). Removal of hemicellulosic fraction exposes the layer of cellulose that enhances enzymatic availability to its microfibrils. This treatment avoids the addition of the acid catalyst and benefits most when applied to the big-sized wood chips.

To enhance sugar recovery, Alvira et al. (2010) recommended a coupled pretreatment. Here, the temperature is kept low to solubilise the hemicellulosic part and then the temperature is increased to 210 °C to solubilise the cellulosic part. Allegedly, the benefits are like higher ethanol productivity and a lesser amount of enzyme needed for enzymatic hydrolysis. However, Maurya et al. (2015) argue that economic aspects of the process must be examined to make the steam explosion process practically applicable.

5.2.2.3 Liquid Hot Water (LHW) Treatment

LHW is yet another hydrothermal pretreatment that generally does not involve quick decompression and any catalytic compound or chemicals. The main purpose of LHW is to increase the cellulose accessibility and to avoid the creation of inhibitory compounds. High temperature (160–240 °C) and pressure is applied maintaining the water in a liquid state to stimulate structural modifications in lignocellulose. To avoid the generation of inhibitory molecules, an acidic pH (4.0–7.0) is maintained. LHW pretreatment is associated with the release of hemicellulosic sugar, mainly in oligomeric forms, so that the undesirable degraded product formation is minimised

mentioned by Maurya et al. (2015). LHW moderately depolymerises and solubilises lignin although its complete removal may not be possible. The major benefit of LHW is that, the solubilised products of hemicellulose and lignin are available in lower concentration. As this treatment has lesser corrosion risk, cheap reactors can be constructed and employed for the purpose.

5.2.3 Chemical Measures

Chemical pretreatment conventionally follows physical/mechanical measures. The physical breakage of the lignocellulose to reduce its size and crystalline nature is further chemically treated to further loosen the chemical bonds and soften it. Chemical measures can further be classified as acid, alkali and ionic treatments or a combination of these.

5.2.3.1 Acid Treatment

A major advantage of acid treatment is it solubilises hemicellulose portion of the substrate. It mostly solubilises xylan hemicellulose converting it to fermentable sugars. Such treatments could be carried out by employing either concentrated or diluted forms of the acids although concentrated forms may generate inhibitory compounds. Also, use of concentrated acids corrodes the reactor and makes the acid recovery process difficult. Thus, dilute acids seem to be suitable and are widely used against a variety of lignocellulose. Reactors like counter-current, shrinking-bed, plug flow, batch and percolation reactors are used for the process. Acid pretreatment is carried out either at high temperature (e.g., 180 °C) for shorter duration or at low temperature (e.g., 120 °C) for longer (30–90 min) duration described by Kumar et al. (2009). Rocha et al. (2009) obtained 0.47 g bioethanol per gram of glucose fermented from cashew apple pretreated with diluted H_2SO_4 at 121 °C for 15 min.

Weak organic acids such as fumaric or maleic acids seem to be good substitutes for enhanced ethanol production. Kumar and Sharma (2017) observed higher glucose release when lignocellulose was pretreated with maleic and fumaric acid followed by hydrolysis. During this pretreatment, usually a mixture of fumaric, maleic and H_2SO_4 is employed. Reportedly, its enhanced wheat straw hydrolysis to generate fermentable sugar with high proficiency mentioned by Mood et al. (2013). However, maleic acid seems to be effective compared to fumaric acid in enzyme hydrolysis for glucose production reported by Kootstra et al. (2009).

5.2.3.2 Alkali Treatment

Alkali pretreatment can be achieved at room temperature in seconds to a few days. This makes it an advantageous proposition. NaOH, KOH, $Ca(OH)_2$ and NH_4OH are usually used in alkali pretreatment, most frequently being NaOH. Zheng and Rehmann (2014) reported a sequential degradation of cellulose, hemicellulose and lignin in lignocellulose through alkali treatment. Alkali pretreatment is more effective against agricultural refuse as against woody materials. Its effectiveness is

associated with the enhanced release of sugars and decreased generation of inhibitors. NaOH pretreatment swells the substrate, thereby reducing its complexity and crystallinity. This stimulates the disruption within the lignin. Kumar et al. (2009) reported 24–55% lignin removal and an overall 14–55% degradation of hardwood by treating it with NaOH. Removal of the most recalcitrant lignin improves the hydrolysis as the cellulose and hemicellulose portions lay exposed for enzymatic action reported by Maurya et al. (2015).

Pretreatment with lime seems still a better proposition due to low cost and safety compared to sodium or potassium hydroxides reported by Maurya et al. (2015). Lime ($Ca(OH)_2$) pretreatment eliminates lignin that enhances the crystallinity index of the biomass. Lime pretreatment eliminates acetyl and easter groups from lignocellulose and improves cellulose degradation. Further, as alkali degrade relatively smaller amount of sugar compared to acids, they are beneficial over acid treatments mentioned by Kumar et al. (2009).

5.2.3.3 Ozonolysis

Selective removal of lignin followed with enzymatic action increases fermentable sugars in lignocellulose. Ozone is a powerful oxidiser for efficient lignin removal. Such treatments, such as ozone pretreatment for agricultural refuse like rye and wheat straws, are done at normal temperature and pressure. Miura et al. (2012) observed that biomass with more than 40% moisture decreased ozonolysis efficiency. Regardless of the few stimulating outcomes, the efficiency of lignocellulose ozonolysis for ethanol production needs further validation.

5.2.3.4 Organosolvs

Pretreatment of biomass with organosolv (organic solvents) is a favourable approach by Alvira et al. (2010), primarily to solubilise lignin fraction. Recovery of relatively undamaged lignin is an important benefit of organosolv pretreatment described by Zhao et al. (2009a). Exposed cellulosic fraction thus formed undergoes efficient degradation enzymatically. Solvents like methanol, acetone, ethyl alcohol, tetrahydrofurfuryl alcohol and ethylene glycol are popularly used. Organic solvents can also be mixed with acid catalysts (such as hydrochloric, sulphuric and oxalic acids) for the purpose to breakdown the hemicellulose bonds. With acid decomposition followed by organosolv pretreatment, achieved up to 70% higher delignification with below 2% loss of cellulose. However, for delignification, an increased (more than 185 °C) temperature may equally be useful in place of an acid reported by Zhao et al. (2009b).

Though organic solvents are useful alternative for chemical pretreatment, these may inhibit the following enzyme hydrolysis and microbial fermentation treatment. Thus, removal of the residual solvent after pretreatment is essential prior to enzyme and microbial fermentation. It can be achieved by simple methods like condensation and evaporation reported by Maurya et al. (2015).

5.2.3.5 Ionic Liquid (ILs)

Utilising such solvent system to pretreat lignocellulose has been extensively used recently. ILs is a salt, characteristically made up of small inorganic anions and large organic cations, which serves well at room temperatures. The solvent characteristics could be changed by regulating anion and the alkyl fractions of cationic form. As there are no harmful gases produced during pretreatment, Alvira et al. (2010) refer to these as ‘green’ solvents. Though the efficacy of ILs to pretreat pure crystalline cellulose has been established, its efficacy over more complex lignocellulosics needs additional studies, although their use in agricultural straw and woody substrate have been reported by Li et al. (2009) and Lee et al. (2009).

Li et al. (2009) obtained 54.8% yield of reducing sugar from wheat straw by pretreating with 1-ethyl-3-methyl imidazolium diethyl phosphate (an IL) at 130 °C for nearly 30 min followed by enzymatic degradation for 12 h. Further, they observed that, the ILs pretreatment didn’t have any negative impact on the sugar fermentation to ethanol by *S. cerevisiae*. However, Alvira et al. (2010) opined that removal of IL moiety is essential to avoid reduction of final concentrations of fermentable sugars as the residual ILs may negatively affect cellulase. Harmfulness of ILs to enzymatic and microbial actions must be considered choosing it for pretreatment. Thus, it calls for additional research for the effectiveness and rationale for the application ILs at commercial scale. Additionally, systems need to be revamped for posttreatment selective recovery of cellulose from the lignin and hemicellulose mixture reported by Hayes (2009).

5.2.4 Physicochemical Measures

5.2.4.1 Ammonia Fibre Explosion (AFEX)

In this method, the substrate is subjected to high temperature (60–100 °C), high pressure and liquid ammonia for a defined time interval. The quick release of pressure ensures further expansion of the gaseous form of ammonia leading to swelling and structure distraction of the substrate fibre. Sarkar et al. (2012) reported enhanced substrate digestibility by this treatment. Kumar and Sharma (2017) successfully undertook AFEX pretreament with ammonia percolation rate (APR) of 5 ml/min maintaining the reactor temperature at 140–210 °C for 90 min. Consequent enzymatic action results in the enhanced release of sugars. A main advantage of ammonia pretreatment is to lower down the formation of inhibitors for the successive biological processes downstream although few phenolic fractions of lignin and supplementary cell wall extracts may persist on the cellulosic surface (Alvira et al. 2010). Agricultural refuse and herbaceous biomass are efficiently hydrolysed through this intervention compared to a highly lignified substrate like woody biomass (Kumar and Sharma 2017). Maurya et al. (2015) opined the need for substrate-specific strategies and operational optimisations of AFEX pretreatment. At optimal conditions, pretreatment could attain up to 90% transformation of lignocellulose to sugars depending on the lignocellulose diversity mentioned by Uppugundla et al. (2014). ARP degrades only hemicellulosic portion and leaves the

cellulosic portion unbroken which results in the formation of short-chain cellulose moiety with high glucan content reported by Alvira et al. (2010). Even though highly volatile, the retrieval and reutilisation of ammonia is possible. However, the difficulty and high expenses in ammonia recovery make it commercially unviable.

5.2.4.2 Wet Oxidation

This technique that utilises oxygen as a catalyst is performed for a short period of time at relatively low reactor temperature suggested by Palonen et al. (2004). Chaturvedi and Verma (2014) carried out the process at 10–12 bar O_2, 170–200 °C, 10–15 min. Oxygen supplementation at high temperature (>170 °C) makes the process exothermic and reduces total energy demand. After simultaneous saccharification and fermentation, it has been extensively utilised for bioethanol manufacturing reported by Martín et al. (2008). Addition of sodium carbonate to the process maintains the pH within neutral to alkaline, thereby decreasing the formation of inhibitory compounds. Wheat straw pretreatment by this process resulted in 96% yield of cellulose fraction and 70% yield of hemicellulose reported by Alvira et al. (2010).

5.2.4.3 Microwave Alkali Treatment

This process involves submerging the biomass in weak alkali and exposing the slurry-like substrate to microwave for 5–20 min mentioned by Keshwani (2009). Alkali is recognised as appropriate chemical components for microwave assisted pretreatment, out of which NaOH is reportedly most effective (Alvira et al. 2010).

5.2.4.4 CO_2 Explosion

Carbon-di-oxide explosion is another common treatment method for lignocellulosic biomass. The process involves exposing the packed biomass to supercritical CO_2 wherein the gas acts as a solvent. Upon suitable increase in the vessel temperature, CO_2 forms carbonic acid at high pressure that degrades hemicellulose. Such pretreatment efficiently removes lignin which in turn enhances the substrate digestibility. Addition of solvents like ethyl alcohol eliminates lignin and further improves delignification suggested by Kumar and Sharma (2017).

5.2.5 Biological/Enzymatic Measures

Pretreating the lignocellulosic biomass with fungi to improve the fodder quality has been established. Currently, this eco-friendly approach has drawn attention as a treatment strategy for enhanced enzyme hydrolysis of lignocellulosic biomass for bioethanol manufacturing. Microbial pretreatment widens the accessibility of the lignocellulose to enzyme degradation. Microbes, mostly white-rot fungi, are employed as agents to degrade lignin, hemicellulose, as well as some amount of cellulose, invulnerable to other components reported by Sanchez (2009). White-rot fungi have been used in ethanol manufacturing process from beech wood with concurrent simultaneous saccharification and fermentation combined with

organosolv pretreatment (Kuhar et al. 2008). Many other fungi, viz. *Pleurotus ostreatus*, *Ceriporiopsis subvermispora*, *Cyathus stercoreus*, *Phanerochaete chrysosporium*, *Pycnoporus cinnabarinus* and *Ceriporia lacerata* have also been employed by Shi et al. (2008) and Kumar et al. (2009). Wheat straw pretreated with *Pichia stipitis* resulted in 0.48 g/g and 0.54 g/Lh ethanol production reported by Kuhar et al. (2008).

Although the foremost disadvantage of biological method is the excruciatingly slow rate of hydrolysis compared to other pretreatments, this pretreatment offers benefits like cost effectiveness, low on energy consumption, no chemicals required and low environmental complication.

5.2.5.1 Enzymatic Hydrolysis

Nonbiological treatments do not completely degrade the hemicellulosic portion of the biomass which calls for enzymatic interventions. Though some delignifying enzymes have been known for partial lignin removal, it is usually resistant to enzymatic decomposition. Studies on the utilising enzymes for pretreatments are a part of inspiring technology for ethanol production in recent times. The enzymes primarily employed for hydrolysis process are discussed below reported by Cheng and Timilsina (2011).

5.2.5.1.1 Cellulase

Usually, the monomeric glucose is the primary substrate for traditional fermentation. Glucose mainly occurs in structure of crystal-like cellulose moieties in plants that comprises of β-1,4 associated glucosyl units. These linkages can be broken by cellobiohydrolases. Activities of cellobiohydrolases can be enhanced by creating new reducing ends by fungal 5-endo-glucanases enzymes reported by van den Brink and de Vries (2011). The β-1,4-endoglucanase and cellobiohydrolases are common cellulose-degrading (cellulase) enzymes in fungi. Although a variety of cellulases is involved in reducing the crystallinity of cellulose, the crystalline property is a major hurdle in its biochemical conversion to ethanol. Nonbiological treatment of cellulose results in the formation of disordered and less crystalline cellulose. This disordered cellulose was more sensitive to enzymatic (cellulases) action rather than dilute acid pretreatment reported by Matthews et al. (2010). Increased binding of cellobiohydrolases to the amorphous region of cellulose resulted in 24-h delay in bioethanol conversion compared to crystalline region.

5.2.5.1.2 Hemicellulase

Xylose is the second most available sugar after cellulose in lignocellulose excluding the softwood that contains xylan support for hemicellulosic fraction. Cellulose microfibrils hold to each other loosely through numerous cross-linkages of glycan molecules (hemicellulosic fractions) by means of hydrogen and carbohydrate bonds mentioned by Gorshkova et al. (2010). The other less-abundant hemicelluloses are xyloglucans and glucomannans and are as such very less in the cell wall. Little research is reported on the impact of hemicellulases on these hemicelluloses. The

essential enzymes to decompose xylan backbone are endo-xylanases like glycoside hydrolase (β-1,4-endoglucanase and β-1,4-endoxylanases) enzymes.

5.2.5.1.3 Accessory Enzymes

Although much of the sugars present in plant cell walls are in form of xylan and cellulose, some other secondary carbohydrates are also present. Depending on the plant species, the secondary polysaccharides comprise of glucomannans and xyloglucans. *Trichoderma reesei* produces glucose releasing enzyme xyloglucanase. Blended with cellulases, xyloglucanase enhanced the conversion rate reported by Benko et al. (2008). However, this advantage is due to the additive effect, or inducer effect of the enzyme is not yet ascertained. Arabinofuranosyl repeatedly cross-link to xylan backbones of lignin through feruloyl ester components. In corn stover, chemically derived xylan is recognised as basic composition with 2-O-/3-O-monoacetyl and arabinofuranosyl components reported by Naran et al. (2009). Families of feuroloyl esterase show strong priority to 2-O-linkage molecule reported by Van den Brink and de Vries (2011). Degradation of arabinofuranosyl basically needs α-arabinofuranosidases enzyme found in fungi. The methyl-glucuronosyl bonds can be broken by α-glucuronidases.

5.2.5.1.4 Synergy Between Xylan Removal and Cellulases

Although the interaction of hemicellulase as accessory enzyme with cellulases is known in some detail, it is not well-known yet. Investigations revealed that pure hemicellulases and cellulases application partially degraded hemicellulosic constituent in lignocellulose. After pretreatment, some hemicellulose still remains in the lignocellulose biomass. Removing the remaining xylan and partially degraded xylan could enhance cellulose hydrolysis. Naran et al. (2009) mentioned that α-glucuronidase and α-L-arabinofuransidase enzymes cleaved the side chain sugars of xylan backbone, thereby maximising the hemicellulose conversion to monomers. Selig et al. (2009) reported removal of sugar side chain arabinofuranosyl by arabinofuranosidases that improved hydrolysis of xylan backbone of arabinoxylan. The acetyl side groups and furyl esters associated with arabinofuranosyl side chains also show direct impact on xylan hydrolysis.

5.3 Cellulosic Ethanol Production

Microbial fermentation is the biochemical conversion of sugars to bioethanol and CO_2. Cellulosic substrates could be transformed to bioethanol by two different procedures mentioned by Amarasekara (2014). Separate hydrolysis and fermentation (SHF) involving two treatments in series in different vessels (cellulosic substrate is enzymatically hydrolysed to degrade the substrate to 5-C and 6-C sugars in the first vessel, and the resulting hydrolysate is microbially fermented in the second vessel wherein the 5-C and 6-C sugars convert to bioethanol) and simultaneous saccharification and fermentation in which the pretreated biomass is enzymatically and microbially fermented in a single vessel (performed either by a consortium of

hydrolytic and fermentative microbes or by using genetically modified microbes that can perform both hydrolysis and fermentation).

5.3.1 Separate Hydrolysis and Fermentation (SHF)

In this, as discussed above, the substrate is subjected to hydrolysis and fermentation in separate vessels. The foremost benefit of this procedure is that, individual stage can be treated at its optimum functioning potential. Its long processing period, however, is the major bottleneck which may inhibit ethanol production and also possibly contaminate reported by Szulczyk et al. (2010). The liquid fraction (hydrolysate) of the enzymatically degraded lignocellulose contains pentose and hexose sugars like glucose, mannose, xylose, galactose, arabinose, fucose, rhamnose, etc. Theoretically, 1 tonne of 6-C polysaccharides (like glucan, galactan or manan) yields 1.1 tonne of hexose sugars. The fermentation of 1.1 tonne of hexose sugars can potentially produce 172 gallons of bioethanol reported by Amarasekara (2014). As per another theoretical estimate, 1 tonne pentose polysaccharides (like xylan and arabinan) results in the generation of 1.14 tonne of pentose sugars which can produce 176 gallons of bioethanol after fermentation.

5.3.2 Simultaneous Saccharification and Fermentation

This procedure is usually helpful in enhancing ethanol production. This is due to the fact that the procedure has no inhibition effect on the hydrolysis end-product. Another benefit of this is the usage of one vessel for both steps. The drawback, however, is that a change in ideal conditions of the temperature of enzymatic hydrolysis and microbial fermentation can disturb the production. Alternately, commercially stable genetically modified hydrolysing as well as fermenting microbes can be employed. However, still this technology is in developing stage.

5.3.3 Microbes in Fermentation

Fermentation of sugar can be done by fermentative microbes like fungi and bacteria. The use of yeast (mainly the Baker's yeast) in fermenting the sugars into ethanol is the first classical technique. Yeasts are eukaryotic fungi and are typically single-celled, even though few variants convert to multicellular by establishing a thread of associated budding cells (pseudo-hyphae). The common yeast is identified as the native or wild-type species *S. cerevisiae*. Also, microbes like *Mucor indicus*, *Pichia stipites* and *Candida shehatae* can ferment sugars to ethanol. Few extensively considered bacterial species that ferment sugars to ethanol include *Clostridium thermocellum* and *Z. mobilis.*

5.3.4 Fermentation Using Yeasts

S. cerevisiae is the commonly used microbe to ferment sugars to ethanol. It has been shown to be highly appropriate also to ferment hydrolysed fraction of lignocellulose. Production potentials of different yeast strains are discussed in Table. 5.1, though *S. cerevisiae* is not capable to ferment pentose sugars like xylose from hemicellulose. Thus, the mixture of pentose and hexose sugars is a massive disadvantage in biomass hydrolysate. The capability of *S. cerevisiae* to ferment pentose sugars is not there due to the nonexistence of enzymes that convert xylulose from xylose reported by Balat (2011).

5.3.5 Fermentation Using Bacteria

Bacteria like *Z. mobilis*, *K. oxytoca* and *E. coli* fascinate the fermentation process reported by Kim and Kim (2012). *Zymomonas mobilis* is a Gram-negative bacterium popular for its ethanol production capability, which exceeds that of *S. cerevisiae* in some aspects. It is effective in fermentation of sucrose, glucose and fructose molecule at a rapid rate. This pathway defines a substitute sequence of biochemical reactions changing the sugar to pyruvate by enzymes that are dissimilar from enzyme used in glycolysis mentioned by Maki et al. (2009). The conversion of one molecule of glucose to pyruvate is associated with the generation of one ATP molecule, one NADPH and one NADH molecule. By contrast, this pathway can form two molecules of ATP along with two molecules of NADPH while converting glucose to pyruvate suggested by Maki et al. (2009). The pyruvate then ferments to yield ethanol and CO_2 as the solitary products, as in yeast. The benefits of *Z. mobilis* on *S. cerevisiae* with reverence to ethanol yield and utilisation of high sugar content for maximum ethanol recovery, maximum tolerance of ethanol up to 16% in the solution, and controlled addition of oxygen is not required during the fermentation.

Z. mobilis can produce up to five times more bioethanol volumetrically compared to wild *S. cerevisiae* reported by Maeda et al. (2013). This bacterium is effective in producing bioethanol from fructose and glucose, although the wild *Z. mobilis* strain is unable to ferment pentose sugars, an important product of lignocellulose hydrolysis. Another drawback of this bacterium is it does not tolerate inhibitory compounds like acetic acid and many phenolic compounds. Effective removal of toxic compounds in fermentation is an important aspect in using this bacterium in sugar fermentation.

5.3.6 Simultaneous Saccharification and Microbial Fermentation

Such treatment of the pretreated biomass is useful for single-container conversion of sugars to ethanol. It needs lesser enzyme and has lesser risk of contamination. In several situations, low pH may enhance lactic acid synthesis and high temperature may unfavourably affect fungal growth, while low pH (like <5.0) and high

Table 5.1 Yeast strains employed in cellulosic bioethanol production

Yeast strain	Strain type	Substrate	Sugar concentration (g/L)	Operation conditions	Ethanol concentration (g/L)	Reference
S. cerevisiae RL-11	Laboratory	Spent coffee grounds	195.0	30 °C, 200 rpm, 48 h	11.7	Mussato et al. (2012)
S. cerevisiae MTCC-173	Laboratory	Sorghum Stover	200.0	30 °C, 120 rpm, 96 h	68.0	Sathesh-Prabu and Murugesan (2011)
S. stipitis CBS 6054	Laboratory	Giant reeds	33.4	30 °C, 150 rpm, 96 h	8.2	Scordia et al. (2012)
S. cerevisiae KL-17	Wild-type	Galactose and glucose	500.0	30 °C, 200 rpm, 28 h	96.9	Kim et al. (2014)
S. pombe CHY0201	Wild-type	Cassava starch	95.0	32 °C, 120 rpm, 66 h	72.1	Choi et al. (2010a)
S. cerevisiae CHY-1011	Wild-type	Cassava starch	195.0	30 °C, 120 rpm, 66 h	89.1	Choi et al. (2010b)

temperature (like >40 °C) may be suitable for enzyme action reported by Dhawan and Kaur (2007). With optimal activity at 55 °C and 4.5 pH, cellulase of *Trichoderma reesei* exemplifies a dynamic form. Similarly, *Saccharomyces* cells function well at 37 °C and pH 4.5 reported by Amarasekara (2014). Thus, as it is crucial to use suitable enzymes and microbes that can work in same operational regime (such as pH, temperature and substrate concentration), the enzymes and fermentation suitability are major factors in this reported by Liab et al. (2000). Major benefits of the technique include: (1) sugar consumption by microbes reduces sugar concentration in growth medium which stimulates enhanced hydrolysis by enzymes to maintain the level of available sugar; (2) enzymes requirements are few and far; (3) there is a scope for enhanced product recovery and (4) environmentally sound technology.

5.3.7 Consolidated Bioconversion Process (CBP)

Amarasekara (2014) and Fujita et al. (2004) successfully fermented amorphous cellulosic fraction directly to ethanol. Consolidated bioconversion process (CBP) is a single-reactor single-course procedure. Recently, it is finding appreciation as a promising way for bioethanol production. Here, lignocellulose fermentation and saccharification are performed in a single step with the help of same microbe. As the same microbe performs both hydrolysis and fermentation, this process is cost effective and energy efficient reported by Liab et al. (2000). Attempts are reported on design CBP-microbes based on two approaches: (1) designing enhanced cellulase producing microbe to produce ethanol and (2) engineering an ethanol producer to produce cellulase. Constructing a *S. cerevisiae* cell in similar lines to integrate cellulose-degrading activity in it seems to be a plausible approach.

5.4 Recent Advancements in Cellulosic Ethanol Production and Application

In sustainable cellulosic ethanol production, the parameters that majorly affecting the cost are production of fermentable sugars and utilisation of reducing water. Chemical pretreatment usually needs large quantity of water during and after the process that may result in the loss of fermentable sugars and generation of high amount of liquid and solid waste. Pretreatments for higher ethanol production could include high gravity biomass processing by IL liquid, enzyme hydrolysis and yeast fermentation. This process enhances biomass digestibility by more than 30%, producing 41.1 g/L ethanol. Xu et al. (2016) reported the feasibility study of this process and indicated that the requirement of ILs during the process reduces by about 90% and wastewater is 85% less generated as well as found a 40% cost reduction in cellulosic ethanol production by one-pot cost-effective and eco-friendly biomass conversion process. Tabah et al. (2016) used solar energy–based continuous flow reactor for bioethanol production by *S. cerevisiae* and recovered 8.7% (w/w) ethanol

from 20% aqueous glucose solution with no loss fermentative activity of *S. cerevisiae*.

Bioethanol-blended gasoline provides better combustion compared to pure gasoline in DI (direct ignition) engines. Compared to gasoline, emission of carbon monoxide and unburnt hydrocarbon decreases in ethanol-blended gasoline. Thangavelu et al. (2016) reported 4.26% enhancement in engine torque in single-cylinder water-cooled engine by 60% bioethanol-blended gasoline. They reported increments by 3%, 6% and 2% in brake power, respectively, with 25%, 50% and 75% ethanol-blending.

5.5 Conclusion

Pretreatment of lignocellulose improves the enzymatic hydrolysis and bioethanol production. The methods are primarily categorised into three: physical, chemical and biological. Physical pretreatments include milling, cutting, grinding, heating, mixing and shearing resulting in size reduction and defibrillation. Chemical pretreatments include alkali, acids, ozonolysis, organosolv and ionic liquids (ILs). Biological pretreatments include the use of enzymatic (cellulases, hemicellulases and other accessory enzymes) and microbial entities. Various combined treatments have been found to be more effective than their individual counterparts. Physicochemical pretreatments, for instance, include steam explosion, ammonia fibre explosion, ammonia recycle percolation, wet oxidation, CO_2 explosion, microwave and ultrasound pretreatments. Generally, pretreatment brings about size reduction, breakdown of fibres, biomass swelling, crystallinity reduction, hemicellulose solubilisation, lignin removal, biomass softening and increase in surface area for enzymatic hydrolysis. After pretreatment, enzymatic hydrolysis is carried out by cellulases and hemicellulases to release sugars from cellulose and hemicelluloses. The process is further enhanced by adding accessory enzymes that hydrolyse secondary polysaccharides. Cellulosic ethanol production can be done in two ways, hydrolysis and fermentation occurring in two different vessels (separate hydrolysis and fermentation) known as SHF, or both hydrolysis and fermentation in a single vessel (simultaneous saccharification and fermentation) referred as SSF. Among the various pretreatment methods, physicochemical pretreatments have been found to be the most effective for supporting the enhancement of hydrolysis process towards high sugar recovery. The microbes used in the fermenting sugars to ethanol are mainly yeast (*S. cerevisiae*) and bacteria (*Z. mobilus*, *E. coli* and *K. oxytoca*, etc.). Attempts on designing CBP microbes based on two approaches are suggested, either designing an enhanced cellulase producing microbe to produce ethanol, or engineering an ethanol producer to produce cellulase. Constructing a *S. cerevisiae* cell in similar lines to integrate cellulose-degrading activity in it seems plausible.

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Production of Biofuel from Disposed Food and Dairy Waste

6

Monika Choudhary, Sunanda Joshi, Vartika, Lavisha Rao, and Nidhi Srivastava

Abstract

Waste-to-energy technologies promise to displace organic waste, including wastewater, livestock, and food waste, for energy use while the global population has risen and more countries have industrialized, and the amount of waste that is disposed of or released for environmental use has steadily increased over the last century. Growing demand for fossil fuels worldwide is harmful to human health and leads to the emission of greenhouse gases. Food waste is easily spread around the world without the use of landfills or incinerators. Nonetheless, this food waste is rich in many nutrients and can be altered to value-added possessions, such as biofuels, using suitable technology. Around the similar period, the growing waste production, combined with increasing population besides existing standards, is a universal challenge for waste managing arrangements. Bioethanol is important for economic and environmental applications in the automotive, beverage, pharmaceutical, and other industries. Biological ethanol processing has been carried out using cellulose materials such as cocoa, pineapple and sugarcane waste, coffee husk, and lactose/cheese/whey hydrolyzed strains. However, such waste may also contain naturally occurring pollutants which may adversely affect the soil or water in which it is deposited.

Keywords

Bioethanol · Livestock · Environment · Cellulosic material · Pharmaceutical

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6.1 Introduction

There has never been a greater essential for renewable energy causes that association environmental approachability through biodegradability, low harmfulness, regenerability besides not as much of dependence on petroleum products. One such form of energy is called biodiesel. It may be derived from animal fats, microalgae oils, vegetable oils, palm oil waste products or animal products, and the fried oils used. Chemically, these are acknowledged as monoalkyl esters of fatty acids. The conservative biodiesel processing approach contains acid besides base compounds for the formation of fatty acid alkyl esters. Downstream dealing out costs and environmental issues associated with the recovery and production of biodiesel has contributed to the exploration for substitute production substrates plus methods. Enzymatic lipase reactions may be an exceptional substitute for the development of biodiesel over a progression normally mentioned to as alcoholysis, a type of transesterification response, otherwise interesterification (ester interchange). Protein manufacturing may be convenient in boosting the catalytic effectiveness of lipases by means of biocatalysts for the development of biodiesel.

However, when converted into rivulets such as ingredients in addition to biofuels, these compounds have considerable economic potential. Indeed, many food waste sources have a fat content and high protein plus sugar, making them ideal for enzymatic assessment. Enzymes have advanced specificity and subordinate energy requirements besides enhanced environmental performance compared to synthetic catalysts for chemical transformation, but their deprived constancy and repossession in their home state limits their efficiency (https://www.nature.com/articles/s41538-018-0028-23/45).

Nearly 40 percent of all food is used by one, two sufferers due to fork continuum in the farm (Fig. 6.1). Although the proportion of appetizing food leftover varies geologically, by product in addition via fact within the supply limitation, the percentage of pre-consumer-generated food as well as agronomic waste (i.e., post-

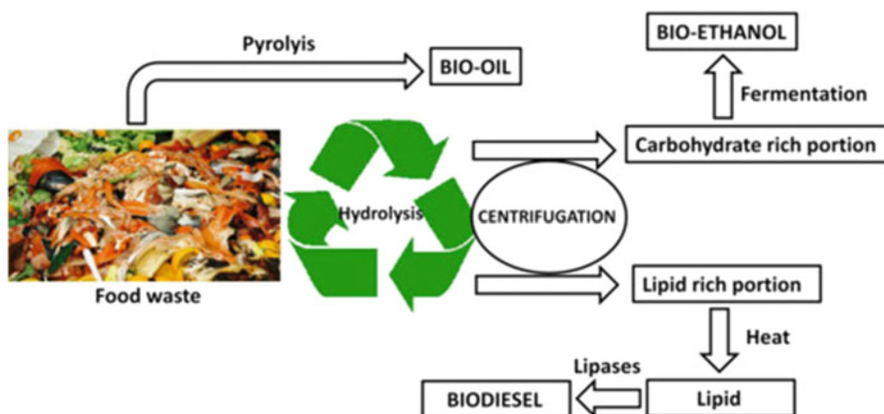

Fig. 6.1 Biofuel production from food waste

harvest and processing) is a major environmental load and remnants a universal problem. By 2050, the world's population is projected to be 9.8 billion, and creative technical approaches need to be advanced to minimize food waste, plus there are substantial food production prospects (UN 2017); indeed, high demand for biological oxygen from food waste streams prevents wastewater treatment plants from being disposed of immediately. While lipids, carbohydrates, and proteins are responsible for their high demand for biological oxygen in food and agricultural waste streams, they can also be transformed into value-added products, converting waste streams into potential revenues streams. Occurrences of major changes comprise phosphorylation, carbohydrate oxidation, acylation, plus hydrolysis, protein deamination in addition to glycosylation, and lipid hydrogenation then esterification. In particular, esterification reactions are commonly used in the processing of a number of value-added foodstuffs besides agronomic products. For biodiesel, oils are esterified through alcohol, sugars may be esterified intended for practice as surfactants, starches may be esterified aimed at custom as biodegradable hot-melt adhesives, plastics in addition to coatings, then flavonoid esterification may improve their bioavailability and effectiveness in the promotion of health and wellness (Alissandratos and Halling 2012; Fang et al. 2002; Walle 2009). Traditional methods to these alterations necessitate a chemical catalytic agent; besides, a substantial energy contribution with restricted response characteristics in addition leads to the establishment of by-products, predominantly in composite conditions such as food waste streams.

There is rehabilitated awareness in, and improved knowledge of, substitutive energy foundations such as bioethanol, hydrogen in addition to biodiesel for usage in diesel engines, particularly in present day that the world's current dependence is profoundly proceeding to petrodiesel or fossil fuel in addition to petroleum (Akoh et al. 2007).

The use of biodiesel is not recent since the diesel engine, i.e., the compressive engine and the inflammation, was first used by Rudolph Diesel for vegetable oil (groundnut/peanut oil) in 1911 (Pinto et al. 2005). Transesterification appears to be the easiest and most costly way of producing biodiesel in large quantities with physical properties similar to fossil diesel and low or no formation of fuel after combustion in diesel engines. Although several scientists have studied the development of biodiesel with chemical and enzymatic reactions (Shah et al. 2003), very few studies have been conducted on biodiesel enzymes (Körbitz 1999).

The enzyme approach is considered to be a "green response" and must be explored at industrial level for the production of biofuels. Recombinant DNA technology will reduce the overall price and pose fewer problems for the downstream processing of biodiesel while generating large amounts of lipase and the use of immobilized lipase then cells (Haas et al. 2002).

In the future, lipase will become the catalytic agent of choice for biodiesel development. It is improved than acid plus base–catalyzed responses, for the reason that it takes not as much of energy and slight or nothing downstream. Base and acid–catalyzed transesterification for neutralization is required. Biodiesel came to prominence because of its environmental profits and the necessity for accurate substitution

of conventional diesel. Yes, biodiesel costs more than petrodiesel for the reason that it comes from exclusive, virgin vegetable oils (Sivakumar et al. 2011). Biodiesel prices are the biggest obstacle to the market (Anuar and Abdullah 2016). Researchers are currently seeking both a low cost and an effective source of biodiesel expansion (Abbas et al. 2016). Biodiesel would reduce its dependence on petroleum fuel. Biodiesel from active slush makes available an opportunity to advance low-cost biofuels (Muller et al. 2014). Although the biological processing plants in the milk processing industry are extremely productive, they produce large amounts of active sludge. The slot is drained and removed separately from the main and secondary tanks. Loams are usually disposed of by incineration in solid waste sites (Kelessidis and Stasinakis 2012). These techniques are economically inefficient and produce secondary waste. The sludge comprises 3–4% solid(s) weighing 4 Wt., adsorbed and ingested milk fat as a means of metabolism. Diverse sectors of food processing that are not reused and inclined of as leftover generate the end product of food waste. There are several sources of food waste taking place at nearly each stage of the food industry and in large quantities (United Nations Industrial Development Organization 2012). Research has revealed that approximately one third of the world's food formed for human feeding is spent annually on approximately 1.3 billion tons of food waste (Gustavsson et al. 2013). People in high-income nations in sub-Saharan Africa are expected to waste nearly equal amounts of food each year as a whole (Gustavsson et al. 2011). Much of the FW comes from customers in the developing world who buy so much and throw away what they do not eat. Mostly due to poor harvesting technology, nonexistence of substructure, manufacturing, and packing conveniences besides unproductive advertising proficiency in developing countries. Recycled feed waste and feed recovery for hungry feeders is the most critical method for managing FW. Nevertheless, more than 95% of food leftover ends in waste dumps (Lin et al. 2013). Furthermore, in direction to encounter the demand of the growing global population, food production would have to be 60% higher than in 2005/2007 by 2050 (Food and Agriculture Organization of the United Nations 2014). The cost of food production has therefore improved suggestively concluded oil prices over the last decade. This means that more and more advanced FW management is required (Ma et al. 2009). Food waste is rich in a variety of organic components, together with 35.5–69% carbohydrates, 3.9–21.9% proteins, organic acids, oils, and fats (Kiran et al. 2014; Lin et al. 2014). The FW can recover and then break down the sugar and protein content into free fermentable sugar plus amino nitrogen (FAN). FW was therefore used to generate various value-added biofuels, products, and bio-based chemicals and enzymes as microbial feedstocks (Ohkouchi and Inoue 2007). In general, using biomass to generate fuel produces more value than using energy and animal fodder. (Lin et al. 2013). There is even more demand on the market for biofuels than for chemical products (Tuck et al. 2012). In accumulation, the FW assessment of biofuels reduces the dependence on rudimentary oil that can alleviate food prices. For this section of the book, we highlight FW for the manufacture of numerous types of biofuels, such as biodiesel, ethanol, hydrogen, and methane. The source-separated HFW was used by *M. thermophilia* as the raw material for the production of extracellular cellulolytic

enzymes. The HFWs used in this study encompassed solvable sugars (sucrose, fructose, glucose, etc.) and insoluble carbohydrates, but no starch, unpredictably. During growth and cellulase secretion, this may be used as a source of nitrogen. Two factors concerning the development of enzymes were estimated and enhanced by *M. thermophila*, specifically the consequence of adding an external source of nitrogen and the HFW attentiveness.

6.2 Biofuels Production

6.2.1 Production of Biodiesel from Food Waste

Biodiesel, an ester produced from monoalkyl fatty acids otherwise fatty acid methyllus ester, is a safe and fresh substitute for petroleum and is therefore a growing sector in the production of biodiesel (Yaakob et al. 2013; Karmee and Lin 2014). Biodiesel from makeovers by way of FW (Fig. 6.1) and waste oil is low-emission, biodegradable and carbon neutral (Yaakob et al. 2013; Wan Omar and Saidina Amin 2011). It is actually one of the most striking substitutive fuels in the world. The EU and the UK have recently seen a considerable increase in the production of biofuel from fats extracted (Lin et al. 2013). There are many techniques for processing FW biodiesel, together with unswerving catalyst or enzyme transesterification and microalgae fermentation.

Conditions that have been established to promote the production of the cellulolytic enzyme *M. Thermophila* was used to generate sufficient quantities of extracellular broth to be used for HFW scurrying. Subsequently concentration of the broth in direction to rise the action per milliliter (from 0.28 FPU/mL initial activity to 3.98 FPU/mL after concentration), HFW was sucked at an preliminary concentration of 30% w/v. Extra fermentation was performed without enzyme in order to evaluate the effect of the addition of cellulolytic enzymes on the production of ethanol at the same solid level. When enzymes were applied, the ethanol yield reached 19.26 g/L after 21 h of fermentation, resulting in a volumetric efficiency of 0.92 g/L•h (Fig. 6.2). The development of ethanol exceeded 38.6% on the basis of solvable besides insoluble, technically possible. The same value was 102.7% on the basis of soluble carbohydrates alone (Matsakas and Christakopoulos 2015).

6.2.2 Production of Bioethanol from Food Waste

Ethanol can be used as a conveyance fuel for the replacement of gasoline, chemical feedstock, heat-burning fuel generation, thermochemical fuel cells, and cogeneration systems. Due to its large industrial applications, the global request for ethanol has recently improved. Bioethanol has traditionally been derived from starchy crops such as corn, potatoes, rice, and cane (Kiran et al. 2014). Ethanol as a gasoline substitute is inadequate since it is also edible in Brazil (sugarcane) feedstock, which is typically used in the US for the production of ethanol (corn) (Lin et al. 2013).

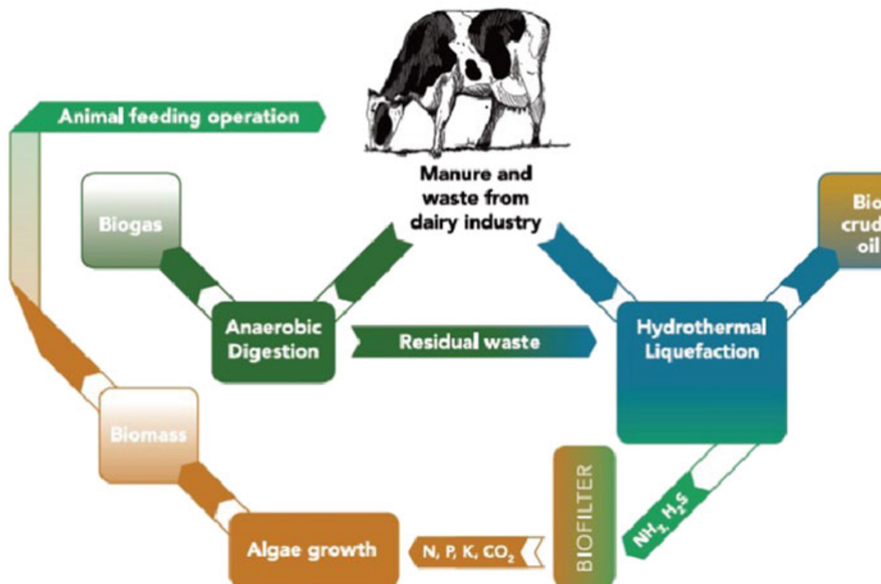

Fig. 6.2 Production of biocrude oil from dairy waste

Maize prices have risen over the last decade as fuel ethanol is produced. As a result, interest in low-cost feedstocks for bioethanol is growing. It has been estimated that "every 100 tons of processed potatoes produce 2–3 tons of starch with a resale value of approximately $180 once it has been recovered" (Kiran et al. 2014). Though, the global production dimensions for bioethanol is only 31 million tons of FW, which represents 61.3 million tons of FW starch consumption.

6.2.3 Pretreatment of Food Waste

There are other issues with the use of FW, such as the exertion of distinguishing between them and the whole waste mass, and the difficulty of being easily destroyed. The rich composition of food waste encourages microbial contamination, contributing to major difficulties in storage and handling. The high water content of food waste generally exists in significant amounts. Dried-up food waste can increase storage stability and reduce total volume. However, FW can be used without pretreatment drying, so that microbial contamination is manageable (Kim et al. 2005). FW also involves complex structural carbohydrates, including cellulose and hemicellulose, though hydrolysis of cellulose is more complicated than hydrolysis of starch. The recovery of FW sugar is even more difficult if cellulose and/or hemicellulose are significantly reduced. Similar FWs have been used as an alternate substrate in some papers for the making of ethanol (Kiran et al. 2014; Pham et al. 2015).

6.2.4 Process Strategies

The conventional method for producing fermentable FW sugar, first by liquefaction or/and sugaring, is separate hydrolysis and fermentation (SHF) and then yeast in ethanol production. High production of glucose due to increased concentrations of enzyme, strong loading, and hydrolysis time is available in the saccharification cycle (Shen et al. 2009; Zhang et al. 2010). However, high levels of glucose can lead to enzyme suppression of catabolites (Huang et al. 2015). As a result, Fed batch, continuous fermentation, and SSF methods have been developed to assess glucose levels and to achieve a high level of FW ethanol (Huang et al. 2015). Historically, a fed-batch crop has been used for high-level growth. Unlike the batch group, Yan et al. reported significantly improved saccharification and ethanol fermentation by batch feed configuration. Continuous fermentation may also be used to reduce the risk of catabolic repression. The production of 0.3 g of ethanol/g of total FW solids with a volumetric productivity of 1.18 g/L/h was achieved through a continuous SHF strategy. SHF, SSF, and ongoing SSF processes have also been reported to convert FW into ethanol. Average ethanol production of 0.43 g/g total solids was achieved during the SHF process. Cell production often included continuous fermentation of bioethanol in the form of FW. The ethanol-producing strain of *Saccharomyces cerevisiae* has been immobilized in a 1.8-L column for continuous production of ethanol on corn stems. Total ethanol titer (84.9 g/L) and yield (0.43 g/g sugar reduction) were obtained at 3.10 h HRT, while mean volumetric ethanol production at 43.54 g/L/h was 1.55 h HRT. Cellulose hydrolysis was the support of the maize stalk used in this analysis. Such a preventive procedure not only increased the efficiency of cell immobilization but also disturbed the rough and porous smooth surface that enhanced the production of ethanol.

6.2.5 Large-Scale Ethanol Production from FWs

Bioethanol production has been developed and is currently being carried out in pilot and larger waste plants. In addition to a pilot study conducted by the China Academy of Sciences, Huainan Normal University found that a 1000 L pilot could produce 44.8 L of ethanol (85% v/v) from a ton of FW. Production of ethanol and volumetric performance in the laboratory or semi-pilot scales were 0.48 g/g, 1.79 g/L/h, respectively. Nonetheless, during the scale-up process, simple models were built to predict fermentation kinetics. An ethanol plant with a dry mass yield of 235 L/t has been developed in Spain to convert citrus waste into bioethanol. ST1 Biofuel has established a network of 11 million liters of ethanol per year from seven plants in Finland. The University of Kumamoto and Hitachi Zosen estimated that a ton of MSW would yield 60 L of ethanol from households and offices. Residual by-products may also be used for biogas production to dig deeper into FW restoration and greener the entire process. According to this chapter, 10 tons of FW from a plant designed by Nippon Steel Engineering Co. have been converted to 400 L of ethanol per day since February 2007. Aemetis Inc. in California permitted the

production of ethanol with a separate annual capacity of 208 million FW in the United States. E-fuel, a California-based company, has developed a home ethanol system for households and small enterprises. The aim of this instrument is to convert ethanol from a microsensor to FW sugar/a starch-rich liquid.

Theoretic estimates are based on Kiran et al. The annual bioethanol capacity of 36, 127, and 593 TL ($\times 10^{12}$ L) is feasible for production in South East Africa, Asia, and the world, respectively. When combined with the processing of additional value-added components in existing food supplies, such as limonene and orange waste extractions, the treatment of waste streams for the production of bioethanol can be economically feasible and can minimize and rehabilitate FW.

6.3 Production of Biofuel from Disposed Food with Enzymatic Approach

The use of enzymes in the food industry can address the issue of food quality. Effective nutritional improvement is enhanced by biotechnological components such as taste, stencil, color, fabric, appearance, and nutritional value. It is estimated that one third of food is lost between fields and forks. They are classified as food waste, both before and after consumption, but there is a lack of understanding which distinguishes data from food waste on a global scale. SSF combines enzyme and ethanol fermentation with low concentrations of enzyme-derived glucose in order to minimize the possibility of substitution of catabolites. For example, this integrated cycle can be achieved through a single container with lower energy consumption, higher ethanol content, and lower enzyme production. Economic growth and governance have two main components: food and electricity. As a result of the doubling of demand for fuel and the overuse of fossil resources, new types of food production, i.e., modern farms, use 100 times or even more fuel than conventional agricultural methods. Food waste management, including conventional and modern technology, (1) supplies fuels and energy that can minimize fossil fuel needs; (2) reduces diversionary food for fuel and animal feed; and (3) the production of biodiesel for food waste (Table 6.1).

6.4 Biofuel Production from Dairy Waste

A paradigm change has resulted in the use of the dairy waste in recent years. Dairy waste crust is produced by dairy productions that knob chilled and raw milk and dairy foodstuffs such as ghee, butter, yogurt, cheese, and ice cream. A huge dairy processing 5 lakh of milk per day produces 250–300 kg of run-off froth per day, making it problematic to dispose of it. Dairy froth is a lesser amount of compact, fluctuating solid mass created by a combination of proteins, fats, lipids, etc., which generates handling complications and processes in handling plants for effluents. Since these scum materials encompass a significant quantity of triglycerides and are consequently used as feedstock. The key purpose of this is to make available an

Table 6.1 Microbial enzymes and their functions

Enzyme	Microorganism	Function
Amylase	*Aspergillus* sp., *Bacillus* sp.	Flour adjustment, bread softness
Maltogenic	*Bacillus stearothermophilus*	Enhance shelf life of breads
Xylanase	*Aspergillus niger*	Dough conditioning
Lipase	*Aspergillus niger*	Dough stability and conditioning
Transglutaminase	*Streptoverticillium* sp., *streptomyces* sp.	Laminated dough strength
Cellulase	*Aspergillus niger*, *Trichoderma atroviride*	Fruit liquefaction
Amylase	*Bacillus*, *Aspergillus*	Starch hydrolysis
Protease	*Aspergillus niger*	Restrict haze formation
Limoninase	*Aspergillus niger*, *A. oryzae*	Debittering
Naringinase	*Aspergillus niger*	Debittering
β-Amylase	*Bacillus*, *Streptomyces*, *Rhizopus*, *β-Glucanase*	Starch hydrolysis

economically practicable and feasible technology for low-cost biodiesel production via waste materials as catalyst and feedstock. The usefulness of the eggshell-derived CaO is being deliberate against the dairy scum transesterification. The consequence of reaction limits such as catalyst setting, reaction time, temperature, and amount of methanol on the yield of fatty acid methyl esters (FAME) was evaluated. The physicochemical features of the produced biodiesel were determined and the performance of the scum biodiesel was compared with conventional diesel, along with its emission characteristics. Using CaO as a diverse catalyst as of eggshell waste in converting dairy scum in the direction of biodiesel may thus boost sustainability over value-added product production.

6.5 Production of Biofuel from Disposed Dairy Waste with Enzymatic Approach

For all species of human and mammalian descent, milk is a natural, complete food. It includes nutrients including fats, proteins, carbohydrates, and vitamins. Many dairy industries manufacture milk and related products such as yogurt, cheese, ghee, milk powder, paneer, ice cream, and other products. Dairy industries handle the number of milk processing, handling, storage, packing and transport equipment, and its products (Fig. 6.2). The list of saccharifying enzymes also included cellulases, xylanases, and pullulanase. To improve cereal hydrolysis for the translation of cellulases and xylanases, starches to glucose can break down hemicellulose in addition to cellulose, respectively, whereas pullulanase can precisely catalyze the α-1,6-glucosidic bond hydrolysis ensuing in linear oligosaccharides being released (Fig. 6.3).

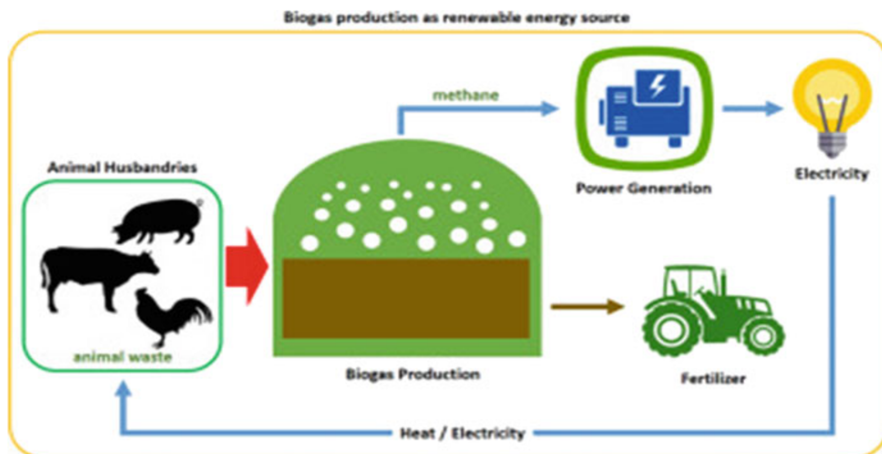

Fig. 6.3 Biogas production from animal waste

6.6 Characteristics and Management of Food and Dairy Waste

Food waste volumes in standard- and high-income provinces are advanced in the downstream process of the FSC, nonetheless only the conflicting trend is found in low-income provinces. Thousands of diverse sustenance products are wasted universally besides each one of them is very difficult to measure. The FAO, however, splits FW into eight possessions together with mueslis (excluding beer), oil, prudish roots and fish and seafood, pulse crops, meat, eggs, milk (excluding butter), fruit (excluding wine), and vegetables (Food and Agriculture Organization of the United Nations 2014). In addition to contributing the large amount of FW, these three regions also contribute significantly to the soil and loss of water, along with greenhouse gas (GHG) emissions from food waste (Food and Agriculture Organization of the United Nations 2014). Among these, commercial restaurant mixed food waste encompasses 33% carbohydrates, 10% proteins, and 15% lipids (Pleissner et al. 2013) (Table 6.2).

6.7 Common Food and Dairy Waste Managements

Food waste management tracks the general outline of managing municipal solid waste together with collection, processing, planning, recycling, and throwing away FW materials. The waste pecking order has been used in many countries as:

1. Preclusion of food waste.
2. Improving sustenance to feed starved people.
3. Providing food for cattle (e.g., pigs, poultry).

Table 6.2 Function of enzymes produced by microorganisms

Enzyme	Microorganism	Function
Transglutaminase	*Streptomyces* sp.	Protein cross linking
Catalase	*Aspergillus niger*	Cheese processing
Acid proteinase	*Aspergillus* sp.	Milk coagulation
Neutral proteinase	*Bacillus subtilis*, *A. oryzae*	Faster cheese ripening, debittering
Lipase	*Aspergillus niger*, *A. oryzae*	Faster cheese ripening, flavor customized cheese

4. Composting and using the compost for soil fertility enhancement.
5. Incineration or else landfilling.

The elementary concept is to evaluate the diverse waste management solutions conferring to their attractiveness for the environment. Food waste hierarchy top of the list is food waste reduction and food repossession to feed starved people. In this circumstance, the food must be edible for certain matters, together with society, sanitary conditions, and ethics.

6.8 Generation of Hydrogen and Methane from Feed Waste

6.8.1 Hydrogen Production

Hydrogen (H_2) is used as a firmed gas and, due to its high energy yield (142.35 kJ/g) and non-polluting combustion, potentials to be one of the utmost significant sources of fuel in future. In addition, the evolving methods for renewable biohydrogen making have been discovered and consist of solar thermal water excruciating, photo electrochemical-splitting water, fermenting sugar-rich feedstock, and transforming renewable resources. A good alternative for H_2 production is the carbohydrate-rich and low-cost FW. Specific fermentation systems have been produced for the development of H_2 on or after FW through hydrogen yields fluctuating from 0.87 mol H_2/mol hexose to 8.35 mol H_2/mol hexose, such as the batch, semicontinuous, continuous, and one or more stages in different scales. Various factors, such as FW composition, FW besides inoculum pretreatments, and progression configurations together with container form and fermentation stage, may affect H_2 development.

6.8.2 Substrate Composition

Various FW conformations vary significantly on or after one another. Enterobacter and Clostridium are the most important FW hydrogen production microorganisms which catabolized sugar but not protein or fat as the source of carbon. Though,

carbohydrate-based waste has greater potential for H_2 production than fat-based and protein-based waste. The FW feedstock carbon/nitrogen (C/N) ratio may reflect the FW content of carbohydrates and proteins. The C/N ratio has recently been reported as a crucial parameter in H_2 manufacturing progressions. While proper nitrogen supply may optimize microbial growth and improve the efficiency of the reactor, its excessive addition can cause ammonia inhibition. Previously, H_2 yield was maintained at approximately 0.5 mol H_2/mol hexose at a C/N ratio of 10–20, while H_2 yield decreased at a higher C/N ratio, escorted by improved lactate, valerate, and propionate production. In an analysis, Chen et al. (2008) also noted that the overall optimum C/N ratio value was 20 to preserve the stable efficiency of solid waste anaerobic digestion (AD). But for various processes and sources, the optimal C/N ratio varied. For example, the optimum C/N ratio is 25 for efficient hydrogen formation by rice straw and coding of wastewater sludge.

6.8.3 Pretreatments

The pretreatment process is at all times needed throughout FW renovation into biofuels. Pretreatment will change FW's physical properties, increase the yield and purity of the product, and avoid microbial contamination and putrefaction. Pretreatment will alter FW's physical property, improve the yield and purity of the drug, and prevent microbial contamination and putrefaction. For biological processes, these should be fragmented by sufficient pretreatment into fermentative and simple sugar and FAN. Pretreatment processes vary between different substrates. While a significant number of reports premeditated H_2 production by means of pure cultivation, assorted crops (e.g., seed sludge) were used to produce H_2 from waste materials. The microorganism practiced for the creation of H_2 in anaerobic digester sludge produced not individually hydrogen producers such as Enterobacter besides Clostridium nevertheless also hydrogenotrophic bacteria. Seed sludge typically is pretreated by heating system to remove consumers of hydrogen. Seed sludge's heat treatment was usually carried out at 90 °C for 15–20 min. The trades of hydrogen were deactivated following heat treatment, although the *Clostridium* sp. foremost because of spore formation. Shin' South Korean company has directed comprehensive work in this extent. FW can similarly be secondhanded as a tool for H_2-producing microflora which can be used to generate H_2 deprived of inoculum subsequently pretreatment.

6.9 Methane Production

Biogas, mainly methane (CH_4), is a kind of renewable energy practiced by humans for a long time. Methane is correspondingly recognized in landfill as a significant source of GHG from FW. Bioconversion of FW into regulated methane not only diminishes the increase in GHG from FW nevertheless too restores the accessible energy as of FW. Additionally, the method of methane extraction can be united

through a two-stage arrangement to produce hydrogen, besides the digestate rich in nutrients can similarly be used by way of a soil conditioner or fertilizer. Anaerobic digestion (AD) can be used to treat almost any organic material, together with grass cuttings, food waste and cardboard, waste paper, industrial effluents, wastewater, discarded food, and animal waste. The consumable FW contains methanogens which make methane making from FW more feasible. Thousands of literature items about the study of methane production by AD method have been written. The key factors influencing the efficiency of AD are close to the hydrogen production cycle, inoculum, feedstock conformations, and cycle conformation.

Combined two-stage hydrogen/methane fermentation anaerobic digestion comprises of acetogenesis, acid regeneration, methanogenesis in addition hydrolysis allowing two-stage compartmentalization in two separate reactors, producing hydrogen and methane. Combined two-stage hydrogen/methane fermentation anaerobic assimilation comprises of hydrolysis, acid regeneration, methanogenesis besides acetogenesis allowing two-stage compartmentalization in two separate reactors, making methane besides hydrogen. Massanet-Nicolau et al. contrasted single- and double-stage anaerobic fermentation systems for biogas production from FW. The yield of methane in the two-stage fermentation was increased by 37%, and the cycle was much more efficient at much shorter HRTs and higher load levels. Han and Shin developed BIOCELL, a novel two-stage process for producing hydrogen and methane from food waste using phase separation, reactor rotation, and sequential batch technique. Two key parts of the BIOCELL cycle were included: for production of H_2 and its posttreatment, there are four leaching bed reactors (LBR) and, for development of CH_4, one UASB reactor. In this method, FW was converted by batch fermentation into H_2 in the four LBRs, and then in the UASB reactor with COD materials, the LBR leachate was continually converted into CH4. Effluent from the UASB reactor was recycled as dilution water via the LBR which resulted in water-saving and improved process stability. The BIOCELL progression could develop a high VS load rate of 11.9 kg/m^3/day in H_2 and CH_4, respectively, at 3.63 L/L/day and 1.75 L/L/day. Furthermore, 72.5% of VS could be eliminated, and the posttreatment production could be used as a soil modification. Kim et al. also castoff the methanogenic effluent to hydrogenesis in the two-stage anaerobic digestion process, foremost to a 75% reduction in alkaline dosage and a 48% growth in hydrogen productivity.

6.10 Applications

Worldwide requisition for biofuels is increasing hastily, as there are finites investments for petroleum-based fuel. In this framework, bioethanol plus biodiesel are common biofuels which are commercially accessible in numerous countries. Can generate comestible biomass for biofuels. That, nevertheless, is previously producing deliberation amid civil society affiliates about food versus fuel. Biofuels from nonedible waste materials also need to be synthesized. Food and dairy waste can be used as a tool for biodiesel and bioethanol processing, because it contains large

quantities of carbohydrates plus lipid also. Industrial bioethanol production plus production of biodiesel as of food waste would help to tackle potential problems of waste management, energy insufficiency, and protection of energy (Karmee and Lin 2014).

6.11 Conclusions Besides Forthcoming Proclivities

Food waste reflects not individual food waste nevertheless then as well soil, water, electricity, and biodiversity waste as well as GHG emissions. Both dairy waste and FW are now causing major cultural, environmental, and social issues. Though, FW is predictable in entirely the food supply chain stages. The environmental harm caused to the existing FW organization by groundwater uncleanness and emissions of GHG, particularly FW superfluous in landfill, should be mainly circumvented. The valorization of FW hooked on biofuels can also associate with FW regulation by energy repossession. This chapter concise the bioconversion of dairy waste and FW into oil in relation to methane, ethanol, biodiesel, and hydrogen. Subsequently, the repossession of nitrogen as well as carbon source, in addition fat was used by way of feedstock to generate biofuels for microorganism. The sustainable method and added value products which lead to the production of biofuels from dairy and food waste become reasonable. In addition, quite a lot of preliminary plants otherwise full-size plants have been mounted in diverse nations, representing that production of biofuel for these waste is technologically also economically worthwhile.

While FW is low otherwise no cost, the costs besides complications of collecting and transporting FW are a concern. Sometimes, the bioprocess is time overwhelming too, through low efficiency. To tackle these issues, it is evident that escalating analysis in addition optimization trainings must focus on assimilating various production processes in addition to value-added products. For example, the fat percentage is used for production of biodiesel, besides the protein plus carbohydrate are added to production of organic acid, exclusively to chemicals network, along with lactic acid in addition to succinic acid, hydrogen, and biogas. In order to accomplish a lesser amount of wasted economy in addition to a more maintainable biobased civilization, this multidisciplinary method will permit us to achieve the wanted approach to managing those waste. Furthermore, letting people know how extreme the problems we face with dairy and food waste should be emphasized. Regulation to minimize the expanse of food superfluous and amount discarded must be implemented besides enforced. Finally, good support for test center- to plant-scale exertion proceeding the FW valorization interested in biofuels would be provided from administrations and companies.

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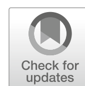

7 Role of Enzymes in Synthesis of Nanoparticles

Swaroop Sanket and Swagat Kumar Das

Abstract

In the last few decades, nanotechnology has come up as an emerging and accelerated interdisciplinary field of science. Multifarious applications of the nano-sized objects are usually attributed to the size and shape, and thus, progressing with size-controlled synthesis of nanomaterials is important. Nanoparticles are obtained by either of the three modes of synthesis, i.e., physical, chemical and biological methods. Biological synthesis or the green synthesis of nanoparticles has received huge attention owing to the economics of production and biological compatibility over the other two methods. Several cellular and biomolecular products from microbes and plants have been successfully utilized to obtain nanoparticles derived using metals and non-metals. Enzyme-mediated synthesis of nanoparticles has provided an alternative approach for the synthesis of nanoparticles in a suitable way. In this chapter, we have compiled several plant and microbial enzymes utilized for the synthesis of nanoparticles.

Keywords

Green synthesis · Nanoparticles · Enzymes · Limitations · Factors

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7.1 Introduction

The application of nanoparticles (NPs) has increased many folds in recent times across different fields of industry including agriculture, health, bioengineering, textile, chemical, paints, etc. Therefore, several approaches are being undertaken to synthesize NPs in a more economic and convenient way. Since size, shape, morphology and stability are few important characteristics in affecting the utilities of NPs, the synthetic approaches should be flexible enough to accommodate the required changes as per needed. The synthesis of NPs can be broadly classified into two types such as top-bottom and bottom-up approaches. The top-bottom approach involves breaking down of suitable bulk material into smaller fine particles by size reduction techniques. Similarly, bottom-up approach involves assembling of atoms and nuclei to grow into required nano size (Gour and Jain 2019). The top-down approach involves different physical techniques like thermal ablation, milling and grinding, microwave (MW) irradiation, ultrasonication, etc. However, the physical approaches are associated with some drawbacks like high energy requirement, costlier and low yield (Shedbalkar et al. 2014). The chemical approach of nanoparticle synthesis involves different approaches including photochemical reduction and electrochemistry techniques. Though chemical approach techniques involve reduced energy during step reduction and production of homogenous particles having high preciseness in size and shape, the methods are not environment friendly and the so-obtained NPs are toxic, unstable and less biocompatible (Kharisov et al. 2016; Shah et al. 2015). Hence, the development of environment friendly technique in which the size, shape, surface charge, stability and characteristics of NPs is one of the most sought research areas in recent times. In this connection, green nanotechnology provides a promising and effective alternative route for NP synthesis. Nanoparticle obtained through biological technique is an environment friendly approach that exploits biological agents such as bacteria, fungi, algae, viruses and plants (Fig. 7.1). This route provides a nontoxic way for nanoparticle synthesis with diversity physico-chemical properties (Gahlawat and Choudhury 2019). Further, green synthetic routes are attractive, considering their potential to reduce the toxicity level exhibited by NPs.

Recently, enzyme-mediated synthesis of nanoparticle is one of the advancements in the field of nanotechnology. Besides, recently many plant- and microbial-derived enzymes have been reported for their ability to synthesize metallic NPs (Adelere and Lateef 2016). However, very few studies have reported the biochemical and molecular mechanisms of enzyme-mediated nanoparticle synthesis. Therefore, the present chapter reviews various enzymes from plants and microorganisms involved in the production of nanoparticles and their possible mechanisms for fabrication of nanoparticles.

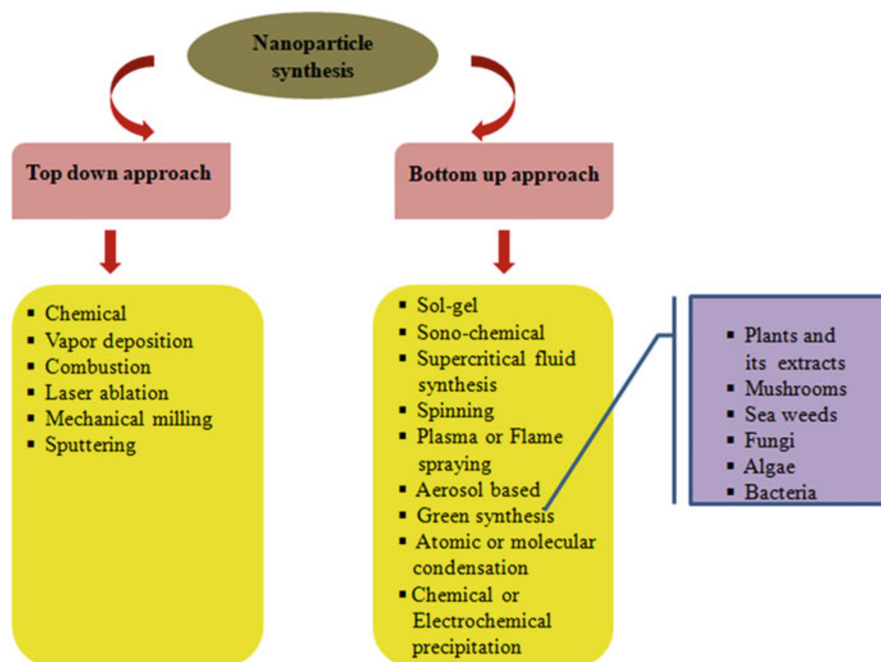


Fig. 7.1 Different approaches for nanoparticle synthesis

7.2 Biogenic Synthesis of Nanoparticle

In green or biogenic synthesis, nanoparticles are synthesized using biological agents such as bacteria, fungi, algae or plants (Ovais et al. 2018). Biogenic methods of nanoparticle synthesis are more suitable as compared to physical and chemical methods which are not eco-friendly and not scalable easily. The plants are composed of an array of complex phytochemicals of different chemical classes such as alcohols, phenols, terpenes, alkaloids, saponins and proteins and can act as a both reducing and capping agents in the biosynthesis of nanoparticles. Similarly, microbes are endowed with different metabolic enzymes which can act as both reducing and stabilizing agents for NP synthesis (Kaushik et al. 2010).

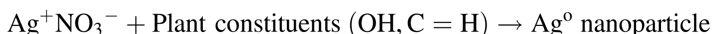
7.2.1 Plants

Biosynthesis of NPs using plant extracts follows a bottom-up approach in which involves synthesis of NPs by using reducing and stabilizing agents (Kalpana and Rajeswari 2018). The phytoconstituents encompass several groups of chemical groups, and hence, the mechanism of biosynthesis of nanoparticles may vary

substantially. Therefore, the mechanism of nanoparticle synthesis using plant extracts has not been elucidated completely. However, metallic nanoparticle synthesis using plants and phytoextracts includes three main phases: (1) activation phase: reduction of metal ions and nucleation of the reduced metal atoms occur; (2) growth phase: the small adjacent nanoparticles spontaneously coalesce into particles of a larger size and (3) termination phase: this phase involves in the determination of the final shape of the nanoparticles (Singh et al. 2016).

Synthesis of nanoparticles using phyto components have generated keen interest in the scientific research community since they are used for the bioreduction of metal ions to form nanoparticles in a more rapid, safer, cost-effective and environmentally safer way. The green approach of nanoparticle synthesis using plant extracts provides a more flexible control over the size and shape of the nanoparticles along with facilitating easy purification. Generally, the metallic nanoparticles are synthesized by incubating the metal salts with the plant extracts. The presence of the various phytoconstituents induces the reduction, and the process is often peaked by several compounds present in the plant cells (phytocompounds) and other reducing agents (Asmathunisha and Kathiresan 2013). The advantages of plant extracts over other biomaterials for synthesis of nanoparticles include easy availability, safety in handling, cost-effective, single-step synthesis process, presence of different secondary metabolites as reductants, rapid rate of synthesis, eco-friendly and stable nanoparticles, size and shape of nanoparticles and suitability for large-scale production (Vijayaraghavan and Ashokkumar 2017).

Several plants and their extracts or bioactive constituents had been explored for the preparation of Ag nanoparticles (AgNPs) in which silver salt (mostly silver nitrate) gets reduced to AgNPs. The reaction is as follows:

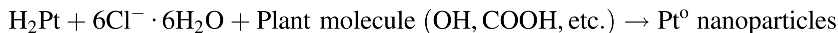


$$Ag^{+}NO_3^{-} + \text{Plant constituents}\ (OH, C = H) \rightarrow Ag^{o}\ \text{nanoparticle}$$

Similarly, gold nanoparticles (AuNPs) are prepared by bioreduction of chloroauric acid ($HAuCl_4$) to AuNPs by plant extracts and the reduction reaction is as follows:

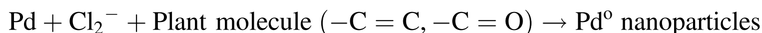
$$H^{+}Au^{3} + 4Cl^{-} \cdot 4H_2O + \text{Plant molecule}\ (OH, COOH) \rightarrow Au^{o}\ \text{nanoparticles}$$

Similarly, the platinum nanoparticles (PtNPs) are synthesized utilizing the plant extracts that reduces the aqueous chloroplatinic acid hexaydrate ($H_2PtCl_6{\cdot}6H_2O$) solution as follows:



$$H_2Pt + 6Cl^{-} \cdot 6H_2O + \text{Plant molecule}\ (OH, COOH, \text{etc.}) \rightarrow Pt^{o}\ \text{nanoparticles}$$

The reduction of palladium chloride ($PdCl_2$) to nanoparticles by plant biomass follows the below equation:



$$Pd + Cl_2^{-} + \text{Plant molecule}\ (-C = C, -C = O) \rightarrow Pd^{o}\ \text{nanoparticles}$$

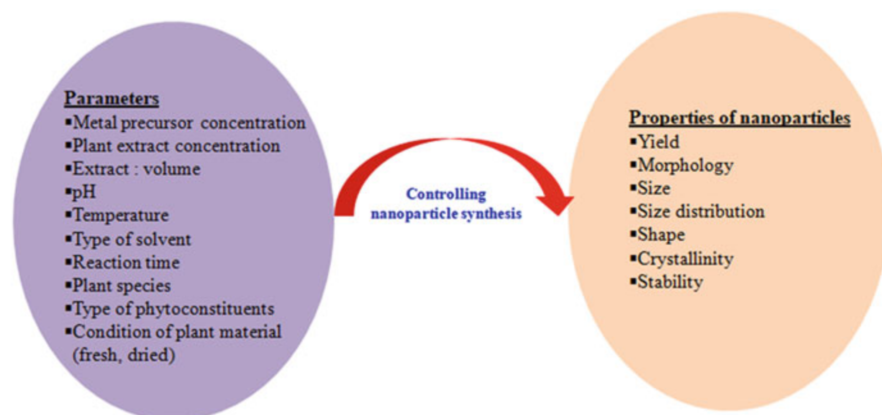


Fig. 7.2 Parameters controlling plant-based synthesis of nanoparticle

Further, several other nanoparticles such as copper nanoparticle, ZnO nanoparticle, titanium dioxide nanoparticle and iron nanoparticle are also synthesized using their metal salts and plant extracts (Vijayaraghavan and Ashokkumar 2017).

Although nanoparticle synthesis using phytoextracts is a surging approach, still few challenges are there, which need to be taken into consideration. Multiple factors affect the plant-mediated synthesis of NPs such as type, source and concentration of plant extracts, ratio of the reagents and experimental parameters like temperature, pH, time, yield and product characterization (Shah et al. 2015; Peralta-Videa et al. 2009) (Fig. 7.2).

7.2.2 Microorganisms

Microorganisms are ubiquitous to almost all environments. Their role in the sustainability of all organisms is extremely important and which is why the wide variety of applications by the use of microorganisms empathizes with its applicability. Their application varies from pharmaceutical sectors to environmental sustainability, further to their use in food industries. Their role in the synthesis of nanoparticles is rather wide and usually attributed to the secretion of metabolites and macromolecules which reduce the metal salts to ionic forms (McDevitt et al. 2011).

Bacteria are omnipresent and are phylogenetically diverse (Sathyavathi et al. 2014; Rohwerder and Müller 2010). The adaptability of the bacteria ranges from highly acidic mine drainage to extreme sub-zero temperature regions (Saeed et al. 2020; Klaus-Joerger et al. 2001; Deobagkar et al. 2015; Alghuthaymi et al. 2015; Feroze et al. 2020). The presence of unique metabolic features in bacteria possess is exploited for the biosynthesis of metallic nanoparticles. Though their exposure to harsh environments leaves them to nothing except cell death; however, with time these unicellular organisms have developed strategies to survive (Gajbhiye et al. 2009; Duran et al. 2015). Several studies have proved that the mineralization of

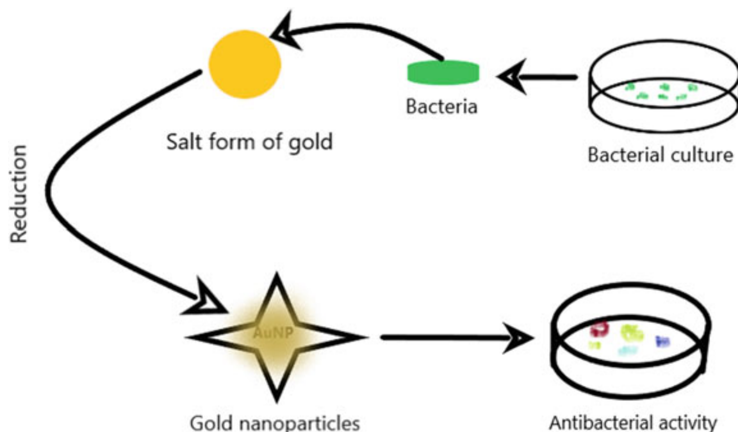


Fig. 7.3 Mechanism of nanoparticle synthesis by bacteria and its application

various metals can be achieved by the use of bacteria (Mishra and Sardar 2012; Kisailus et al. 2005). In a study by Saeed, Iqbal and Ashraf on the effect of bacteria-mediated silver nanoparticles on human pathogens, the silver nanoparticles were able to exhibit antibacterial activity against *Staphylococcus aureus* exhibiting resistance to methicillin (MRSA) and few other drug-resistant strains as well. The zone of inhibition they observed was ranging from 10 to 28 mm (Yang et al. 2016). In another study by Klaus-Joerger et al. (2001), bacterial cells were exploited for the accumulation of biosynthesized nanoparticles. They reviewed the properties of the nanoparticles and concluded that the use of bacteria-mediated nanoparticles can be utilized for structured materials (Zomorodian et al. 2016). Deobagkar et al. (2015) studied the highly resistant *Deinococcus radiodurans* bacteria to synthesize silver nanoparticles. The bacterium was able to accomplish the objective under optimized conditions. The effect of the biosynthesized nanoparticles was tested against for antibacterial and antifouling activity. Further they were also able to inhibit cell proliferation of cancer cell lines (Gholami-Shabani et al. 2015) (Fig. 7.3).

Fungi are an excellent source for the synthesis of nanoparticles. Their capacity is understood from the tolerance levels to different lanthanides and transition metals. The large-scale production of macromolecules especially enzymes makes fungi as one of the suitable biological agents for the synthesis of different metallic nanoparticles (Khan and Ahmad 2014). Feroze et al. (2020) studied the antibacterial activity of fungal nanoparticles synthesized using silver nitrate (Fig. 7.4). They adopted the method of well diffusion to assess the antibacterial effect of the biosynthesized nanoparticles against some of the notorious pathogens. Their synthesized nanoparticles also suggested the efficacy of wound healing and as an anti-inflammatory agent (Kumar et al. 2007). Gajbhiye et al. (2009) in their study on the combinatorial assessment of nanoparticles with fluconazole showed several pathogenic fungi that were inhibited in the presence of silver nanoparticles (Duran et al. 2014).

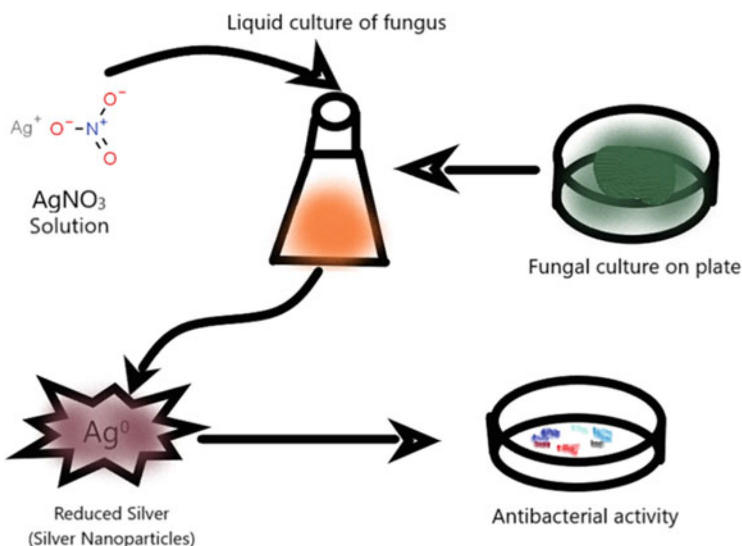


Fig. 7.4 Mechanism of nanoparticle synthesis by fungi and its application

7.3 Enzyme-Mediated NP Synthesis

Enzyme-mediated nanoparticle synthesis is considered as environmentally friendly, economic and easily scaled-up process. Enzyme-mediated nanoparticle synthesis is one of the most promising synthesis strategies in recent times in the field of nanobiotechnology. The enzymes may differently behave during the formation of nanoparticles like reducing and capping agent (Adelere and Lateef 2016; Duran et al. 2014).

7.3.1 Plant-Based Enzyme-Mediated Synthesis

Enzymes by their general characteristics modulate the synthesis but do not involve in the biochemical reactions itself. They may also sometimes serve as reducing and stabilizing agents. Duran et al. (2014) showed the involvement of sulphur-containing groups and disulphide bridge moieties present in enzymes during nanoparticle formation process. Similarly, sulphur moieties of denatured enzymes also help transform the metallic ions to form nanoparticles. Enzymes present in plants may act as catalysts modifying the reduction speed or acting simply as chemicals having a direct reducing activity towards the cation. Several studies have reported plant-derived enzyme-mediated synthesis of nanoparticles which are discussed in Table 7.1.

Table 7.1 Plant-derived enzyme-mediated nanoparticles

Enzyme	Source	Types of nanoparticle	Mechanism of synthesis	References
Amylase	Plants	Ag	Interaction of the thiol group (−SH) of cysteine with the metal ions leading to the reduction of metal ions to corresponding metal atom	Mishra and Sardar (2012)
Cysteine protease	*Calotropis procera*	Cu	Act as capping/stabilizing agent; bind to metal nanoparticles through the free amine groups or carboxylate ion	Dubey and Jagannadham (2003)
Curcain protease	*Jatropha curcas*	ZnS	As reducing and stabilizing agents. Cysteine or thiol residues present in curcain may be donating these sulphide (S^{-2}) ions to Zn ion	Hudlikar et al. (2012)
Peroxidase	*Armoracia rusticana*	Ag, Au	Reduction of $HAuCl_4$ by $NaBH_4$	Parashar et al. (2017) and Kumar et al. (2018)
Urease	*Canavalia ensiformis*	Au, Ag, Pt, ZnO	Urease acts as a reducing and stabilizing agent for the synthesis of nanoparticles	Sharma et al. (2013)

7.3.1.1 α-Amylase

α-Amylase is one of the most common enzymes used in in vitro nanoparticle synthesis. It acts as both reducing agent and capping agent in green synthesis of nanoparticle. Mishra and Sardar (2012) reported the synthesis of silver nanoparticles using α-amylase from aqueous solution of silver nitrate. The mechanism behind nanoparticle synthesis could be attributed to the interaction of the thiol group (–SH) of cysteine with the metal ions leading to the reduction of metal ions (Ag^+) to corresponding metal atom (Ag^o). α-Amylase is also involved in the synthesis of gold nanoparticles. The frees –SH group present in the amylase enzyme helps in the reduction of $AuCl_4^-$ to Au nanoparticles (Rangnekar et al. 2007).

7.3.1.2 Glutathione

Glutathione (GHS) is considered as one of the most common antioxidants present in plant cells. It is a reducing agent and has highly reactive thiol group and hence can be used to convert the oxidation state of the metals. Along with the thiol group, GSH molecule also contains amine and carboxylate functional groups which may help in cross-linking to other molecules. Baruwati et al. (2009) reported the synthesis of AgNPs, PdNPs, PtNPs and AuNPs using glutathione under microwave irradiation conditions. The glutathione acts as both reducing and coating agent in synthesis of metal nanoparticles synthesis.

7.3.1.3 Protease

Cysteine protease isolated from medicinal plant *Calotropis procera* (Family Asclepiadaceae) has molecular weight and isoelectric point of 28.8 kDa and 9.32, respectively (Dubey and Jagannadham 2003). The enzyme has been used to fabricate copper nanoparticles from copper acetate. The proteinaceous material encapping the particles has possibly served capping/stabilizing agent. It has been reported that proteins attach the metal ions in the nanoparticles through the presence of free amine groups or carboxylate ions in the amino acid residues (Harne et al. 2012). Similarly another protease, e.g. curcain isolated from latex of *Jatropha curcas* plant has been reported for the synthesis of zinc sulphide (ZnS). The curcain enzyme present in the latex of *J. curcas* acted as both reducing and stabilizing agents. The cysteine or thiol residues present in curcain may be donating these sulphide (S^{-2}) ions to Zn ions and helps in green synthesis of ZnS NPs (Hudlikar et al. 2012).

7.3.1.4 Peroxidase

Horseradish peroxidise (HRP) obtained from *Armoracia rusticana* has been reported for the synthesis of Ag and Au nanoparticles. Parashar et al. (2017) have reported the green synthesis of AuNPs using $HAuCl_4$ and $NaBH_4$ and HRP at optimized condition. The reduction of $HAuCl_4$ was carried out by $NaBH_4$ and H_2O_2 further speeds up the reduction process. The formation of AuNPs was then mediated by HRP. In another study, Kumar et al. (2018) describe the synthesis of AgNPs using HRP. The enzymatic activity of HRP assisted in the formation of AgNPs, which was prevented upon the addition of an excess amount of hydrogen peroxide (H_2O_2).

7.3.1.5 Urease

Urease isolated from *Canavalia ensiformis* (jack bean plant) has been shown for the synthesis of Au, Ag and Pt nanoparticles. The enzyme acts as a reducing and stabilizing agent. The catalytic activity of urease is also exploited for the synthesis of ZnO core-shell nanostructures at ambient temperature. The exposed residues, i.e. Cys592 in the enzyme, was found to be responsible for the formation of metal and metallic alloy nanoparticles (Sharma et al. 2013). Zn^{2+} binds on the negative charge urease present on the surface through weak bond interaction at a pH of 9, thus forming of zinc hydroxide as an intermediate compound. Under the basic conditions, further dehydration of zinc hydroxide yields ZnO on the enzyme surface accelerated by the “salting out” effect (Makarov et al. 2002).

7.3.2 Microbial Enzyme-Mediated Synthesis

Microbial enzymes play an important role in the formation of metal salts leading to the synthesis of metal NPs. The enzymes act as reducing agents and work as an electron shuttles during the reduction of metals and synthesis of microbial NPs (Subbaiya et al. 2017). Therefore, optimization of conditional parameters for maximizing the activity of enzymes may enhance the synthesis of NPs. Few

Table 7.2 Microbial enzyme-based synthesis of nanoparticles

Enzyme	Microbial source	Type of nanoparticles	References
α-Amylase	*Aspergillus oryzae*	AgNPs	Mishra and Sardar (2012)
Aspartate protease	*Aspergillus saitoi*	AuNPs	Bharde et al. (2007)
Hydrolase	*Tethya aurantia*	Gallium NPs	Kisailus et al. (2005)
Hydrogenase	Sulphate-reducing bacteria (SRB)	Platinum nanoparticles	Riddin et al. (2009)
Laccase	*Pleurotus ostreatus*	AuNPs	El-Batal et al. (2015)
Laccase	*Trametes versicolor*	AgNPs	Duran et al. (2014)
Nitrate reductase	*Bacillus licheniformis*	AgNPs	Li et al. (2011a, b)
Nitrate reductase	*Rhodopseudomonas capsulata*	AuNPs	He et al. (2007)
Sulphite reductase	*Thermomonospora* sp.	AuNPs	Khan and Ahmad (2014)

microbial enzymes involved in NPs synthesis with their sizes are mentioned in Table 7.2.

7.3.2.1 α-Amylase

Rangnekar et al. (2007) studied the biosynthetic process of gold nanoparticles. In their study, the conversion of chloroauric acid was converted to gold nanoparticles by the catalytic action of an amylase enzyme (Rangnekar et al. 2007; Duran et al. 2015). Mishra and Sardar (2012) also synthesized nanoparticles from silver salt using amylase enzyme. The observations made by them was accumulated to the active role of the enzyme amylase which could form the silver nanoparticles from the salts of silver (Mishra and Sardar 2012).

7.3.2.2 Hydrolases

The importance and role of hydrolases in the synthesis of nanoparticles was realized by Ramezani et al. (2010). They reviewed the properties of this enzyme in fungi and reported that the fungal specie *Verticillium* sp. could utilize the enzyme hydrolases to convert $[Fe\ (CN)_6]_3$ and $[Fe(CN)_6]_4$. The enzyme hydrolases was also explored by Kisailus et al. (2005) in their study on gallium salts which was capable of transforming the salts of gallium to gallium (III) nitrate. The particular enzyme was also found to be forming the crystal forms of the metallic nanoparticle at very low temperature.

7.3.2.3 Sulphite Reductase

Zomorodian et al. (2016) synthesized silver nanoparticles using three Aspergillus species. Their UV-Vis spectroscopic analysis showed the absorption at 430 nm which coincided with that of silver nanoparticles. They concluded that the formation

of silver bio-nanoparticles was regulated by nitrate reductase enzyme. Gholami-Shabani et al. (2015) synthesized gold nanoparticles by adopting a cell-free extract from the bacterium *E. coli.* The cell-free extract contained the enzyme sulphite reductase which could exhibit antifungal activity against *Aspergillus fumigatus* and *Fusarium oxysporum* and two other fungal species. Their synthesized nanoparticles were able to show a MIC of 31.25 μg/ml to 250 μg/ml. The utility of this enzyme was also shown by Khan and Ahmad (2014). They described a protocol for the purification of the enzyme sulphite reductase that helped in rendering the gold nanoparticles to disperse into the solution. In another study conducted by Kumar et al. (2007), the role of sulphite-reductase was elaborated. Their study demonstrated that even fungal species (*Fusarium oxysporium*) could produce the enzyme sulphite reductase for the synthesis of gold nanoparticles.

7.3.2.4 Laccases

Duran et al. (2014) utilized the enzyme laccases in a semi-purified form obtained from a fungus *Trametes versicolor*. The authors concluded that the enzyme used for the synthesis of silver nanoparticles was interaction of silver ions with the T1 catalytic site of the enzyme laccases. Lateef and Adeeyo (2015) carried out a study on the efficiency of biosynthesized nanoparticles using laccase enzyme. Their study reports the efficiency of the laccase enzymes in the synthesis of nanoparticles could inhibit few pathogenic bacteria at a higher rate.

7.3.2.5 Nitrate Reductase

Multiple studies reported the involvement of nitrate reductase enzyme in the production of AgNPs by *Bacillus licheniformis* (Kalimuthu et al. 2008; Kalishwaralal et al. 2010). NADH-dependent nitrate reductases enzymes require cofactors like NADH for production of metal NPs. Different studies demonstrated the role of NADH and NADH-dependent enzymes (nitrate reductase) in *Bacillus licheniformis* for the possibility of forming Ag^0 (Duran et al. 2011; Li et al. 2011a, b). Similarly, in another study bioreduction of Au is initiated via electron transferring from NADH by NADH-dependent reductase enzymes present in *Rhodopseudomonas capsulata*. Consequently, Au ions accept electrons and get reduced (Au^{3+} to Au^0), leading to the formation of gold nanoparticles (He et al. 2007).

7.4 Factors Affecting Enzyme-Mediated NP Synthesis

Several factors including the quantity of enzyme, pH, temperature, enzyme to substrate concentration ratio and incubation time of reaction are limiting factors in synthesis and controlling the size of metallic NPs. The following section discusses various factors affecting the enzyme-mediated nanoparticle synthesis.

Phanerochaete chrysosporium derived enzymes like Laccase and ligninase have been reported for the formation AuNPs of 10–100 nm in particle size. Several factors like incubation age of the fungal culture, concentration of $AuCl^{4-}$ solution and temperature affect the shape of AuNPs (Sanghi et al. 2011). He et al. (2007) reported

the synthesis of AuNPs by *Rhodopseudomonas capsulata*-mediated via NADH and NADH-reliant enzymes. Several factors like concentration of the predecessor, pH, temperature and duration of reaction are limiting factors in controlling the size of MtNPs. pH value of the reaction mixture was found to be an important factor for controlling the size and shape of AuNPs. In another study, Riddin et al. (2010) demonstrated the effect of platinum salt concentration (H_2PtCl_6) on Pt NP synthesis. The ratio of H_2PtCl_6 to bacterial hydrogenase enzyme varied from 0.7:1 to 4:1. Amongst them, H_2PtCl_6 to bacterial hydrogenase enzyme at 1.5:1 was reported to be the optimized condition for Pt NP synthesis. One of the important parameter affecting NP synthesis in more economical and efficient way is concentration of substrate. Gradual increase in the concentration of $AgNO_3$ to 5 mM, AgNP production was increased using *Fusarium oxysporum*-mediated enzymatic AgNPs synthesis. However, further increasing to 10 mM, the production of AgNPs decreased (Korbekandi et al. 2013).

7.5 Limitations of Enzyme-Mediated NP Synthesis

In spite of a wide range of benefits obtained from enzyme-mediated synthesis of metal NPs, there exists a number of challenges to overcome. One of the major limitations in enzyme-mediated synthesis is lack of complete and thorough understanding of mechanical aspects of biofabrication of nanoparticles. Detailed analysis of metabolic pathways is required to obtain tailor-made nanoparticles (Ovais et al. 2018). Considering the requirement in biomedical purposes, it remains an indispensable agent owing to biocompatibility of NPs. It is important that nanoparticles remain stable without any significant change of morphology, shape, size and structure (Dauthal and Mukhopadhyay 2016). Surged studies are required to ensure the efficacy and long-term stability of enzyme-fabricated nanoparticles. Large-scale production is yet a major bottleneck for commercialization of enzyme-mediated nanoparticle fabrication along with controlled sizes and shapes. Bulk processing methods for enzyme-mediated nanomaterials and downstream processing techniques also need substantial improvement.

7.6 Conclusion and Future Prospective

There is immense potential for enzyme-mediated metal nanoparticle synthesis as the process is eco-friendly, low in toxicity, less expensive, high biodegradability and are applicable for therapeutic purposes. However, enzyme-mediated green metallic nanoparticle synthesis requires in depth knowledge of the biochemical and molecular mechanisms of the reactions involved during synthesis for a better understanding of chemical composition, shape, size and mono dispersity of nanoparticles. Therefore, detailed studies are required to find out the exact role of enzymes and their optimised reaction conditions required for synthesis, stabilization or pharmaceutical activities of NPs. With improvement of our knowledge, enzyme-mediated

nanoparticle synthesis could be the leading large-scale production method for nanoparticles in coming days. Detailed characterization of enzymes used for the NP formation and biogenic activities could open up a new pool of proficient enzymes which could be utilized for various biomedical applications in future.

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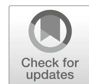

8 Protein–Nanoparticle Interaction and Its Potential Biological Implications

Manoranjan Arakha, Sandip Kumar Rath, Arun Kumar Pradhan, Bairagi C. Mallick, and Suman Jha

Abstract

Nanoparticles in the biological environment appear in different shape and size and inside the biological milieu interact with different biomolecules. Interaction of nanoparticles with protein leads to the formation of dynamic nanoparticle–protein complex also known as nanoparticle–protein corona. However, the protein corona formed at nanoparticle interface might influence different properties of nanoparticles such as cellular uptake, accumulation, inflammation and clearance of nanoparticles. The findings from different studies on nanoparticle–protein interaction rationalized that nanoparticle interface results into conformational rearrangement of the adsorbed protein molecules, affecting the bioreactivity of the nanoparticles. The current chapter discussed on the conformational rearrangement of protein/peptide at nanoparticle interface and its biological applications. Additionally, different possible factors such as size, shape, concentration of nanoparticles and forces at nanoparticles interface affecting protein conformation are also thoroughly discussed. This chapter also highlights some important applications of nanoparticle–protein interactions like nanoparticles as possible therapeutic agents against protein amyloidosis, enhancement of antimicrobial

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propensity of peptides upon interaction with nanoparticles, use of nanoparticles as different biosensors, etc.

Keywords

Nanoparticles · Protein corona · Bioreactivity · Enzyme nanoparticles · Protein amyloidosis

8.1 Introduction

The concept of nanotechnology was first introduced by Nobel physicist Professor Richard Feynman by delivering a speech 'There's Plenty of Room at the Bottom' to the American Physical Society in December 1959. The delivered speech at the conference focused on different possibilities, i.e. if we could understand how to control even single atoms and molecules (Arakha and Jha 2018; Toumey 2009). The outcome of the speech by Professor Feynman led the scientific community to a new era of technology, known as nanotechnology. The basic aim of the technology is to formulate new molecular structures with advanced physico-chemical properties for application in various fields of science and technology. Nanoparticles are considered to be the basic fundamental units of nanotechnology, and the nanoparticles have drawn tremendous attention since they bridge the physical/chemical gap between the atomic/molecular structure and bulk (macroscopic) material (Arakha and Jha 2018). However, the definition of nanoparticles was suggested by National Nanotechnology Initiative (NNI), USA, as material with average size of 1–100 nm in at least one of the three dimensions (Kim et al. 2011). Due to unique features like high surface to volume ratio and improved percentage of grain boundaries, nanomaterials are quite different from macroscopic bulk materials (Arakha et al. 2015a, b; Fang et al. 2006). In fact, nanoparticles as the fundamental/functional units in nanotechnology possess unique physico-chemical properties, since they fall in the transition zone from the atom/molecule to macroscopic material/bulk material. The advanced physico-chemical properties of nanomaterials in comparison to bulk materials are attributed to small size, shape, surface structure, chemical composition, solubility and aggregation propensity in colloidal solution (Arakha et al. 2016, 2017; Nel et al. 2006). Hence, nanoparticles, for their enhanced properties over respective macroscopic material, are being adopted in different fields like drug delivery, diagnostic techniques, disinfectants, antimicrobial bandages, sunscreen, etc. (Meruvu et al. 2011). To complement enormous requirement by various fields of engineering and technology such as drug delivery, sunscreens, cosmetics, paints, fabrics, sporting goods and electronics, engineering of nanomaterials is growing exponentially (Miller et al. 2017; Nayak et al. 2016; Tiwari et al. 2018; Valsami-Jones and Lynch 2015; Yadav et al. 2018).

Proteins, the essential biomolecules, are synthesized on ribosome control most of biological processes inside and outside of a cell. Following the synthesis on ribosome, it folds into three dimensional structures those are further stabilized by

posttranslational modifications in eukaryotes. Hence, the three-dimensional structures of proteins determine the functions of most proteins. However, the native structure can be destabilized by perturbing the network of different interactions, like non-covalent interactions, van der Waals interactions, hydrogen bonds, hydrophobic/hydrophilic effects, electrostatic interactions, salt bridge interactions, dipole–dipole interactions, etc. (Shao et al. 2011). Unfortunately, change in local physicochemical environment of proteins lead to the perturbation of the non-covalent interaction network, taking the conformation from folded to partially or completely unfolded conformation. Protein, in both of the cases, loses their functions resulting in degradation of proteins by proteostasis network of cell (Hipp et al. 2019). However, sometimes these confirmation goes unchecked by proteostasis network, and their accumulation results into the self-assembled pathogenic structures like amyloid fibrils (Bellotti and Chiti 2008; Jahn and Radford 2005). The self-assembly process of monomers is accompanied by many intermediate forms with cytotoxic propensity (Jha et al. 2009; Xue et al. 2009), leading to various degenerative diseases like amyloid polyneuropathy, Huntington, Parkinson, Alzheimer, type 2 diabetes, spongiform encephalopathy diseases, etc. (Bellotti and Chiti 2008). Generally, three types of degenerative diseases are reported associated with amyloid fibrils, such as (1) neurodegenerative diseases like Alzheimer's diseases where amyloid fibrils are degenerated in brain cells, (2) non-neuropathic localized amyloidoses where fibrils cause degeneration of particular kind of cell other than neuronal cells, (3) degeneration of particular kind of cell other than neuronal cells where degeneration of multiple kind of cells other than neural cells were marked (Bellotti and Chiti 2008). However, development of effective drugs against this amyloid disease is an issue for last decade. Although worldwide research is going on, however therapeutic agents to combat/hinder the amyloidoses developed till yet show insignificant effect. Since the insoluble fibrillar deposits which are irreversible in nature are the key cause for misfolded proteins, reversal of these aggregates would be an attractive strategy to formulate therapeutic agents against the protein misfolding disease (Antosova et al. 2012). In this context, first vaccine 'Doblin-based Elan Pharmaceuticals AN-1792' was developed to treat Alzheimer's disease, which was successful in case of mice but not in human. Hence, various research groups worldwide are doing research to formulate effective vaccines against amyloid diseases. Nowadays, the nanoparticles, having advanced physico-chemical properties, have attracted the attention of different researchers to inhibit amyloid fibrillation (Antosova et al. 2012; Bellova et al. 2010; Fu et al. 2009; Rocha et al. 2008).

The nanoparticles exhibit advanced physico-chemical properties in comparison to bulk materials, hence are used in drug delivery, diagnostic techniques, disinfectants, antimicrobial bandages, sunscreen, etc. (Meruvu et al. 2008; Panda et al. 2016; Sharma et al. 2018). Among the various activities of nanoparticles, interaction with protein, forming nanoparticle–protein conjugates have drawn great attention due to its direct or indirect involvement in various applications from sensing, imaging, assembly to control biological processes (Leszczynski 2010; Shang et al. 2007a). Upon conjugation with nanoparticles, protein brings biocompatibility or cytotoxic

propensity to nanoparticle. However, sometimes protein–nanoparticle conjugation leads to major/minor structural change in protein upon adsorption to nanoparticle surface (Shang et al. 2007a). The changes in protein structure on adsorption onto nanoparticle surface result in loss of the protein activity, depending upon the extent of conformational changes brought upon conjugation with nanoparticles. Additionally, conformational changes in protein on conjugation with nanoparticle may help in either enhancing or inhibiting the amyloidogenic propensity, depending upon the interaction pattern at the interface. Thus, the following headings are focused on different physical and chemical nature of nanoparticle interfaces that brings the conformational rearrangements in a protein.

8.2 Nanoparticle–Protein Interactions/Conformational Rearrangement of Protein at Nanoparticle Interface

Nanoparticles, inside the biological milieu, interact with different biomolecules, membrane, protein, DNA, etc. to further reduce its surface free energy content to attain the stability in new physico-chemical environment (Monopoli et al. 2012). Thus, as a result of the interactions, the NPs are properly dispersed in the biological environment. Nevertheless, NP interface, formed inside the biological fluids, forms attractive interactions with different biomolecular surfaces. Thus, the attractive interactions result in interface which acts against the agglomeration of nanoparticle. Among the biomolecular surfaces, the presence of protein at the interface results in complexes known as 'nanoparticle–protein corona'. As described by Monopoli et al. (2012), biological 'corona' formed due to interaction between the NP and biomolecules is considered as elements of biological identity of nanoparticles (Monopoli et al. 2012). However, different characteristics of NP like size, shape, nanoparticle composition, surface charge, surface modifications and solubility play important roles in determining the strength and kind of interaction with different biomolecules, thus the biological response and distribution (Chithrani et al. 2006; De Jong et al. 2008; Dobrovolskaia et al. 2008; McNeil 2005; Tomalia et al. 2007). Although various biomolecules are adsorbed onto the NP interface; however, the formation of protein–nanoparticle complexes has attracted the interest of various research groups as an emerging area of research (Aggarwal et al. 2009; Brown et al. 2001; Dutta et al. 2007; Goppert and Muller 2005a; Kiwada et al. 1987; Lynch and Dawson 2008; Monopoli et al. 2012; Muller and Heinemann 1989; Tyrrell et al. 1977). It is reported that generally 3700 proteins are there in plasma proteome; however, nearly 50 proteins are reported to bind with different nanoparticles (Aggarwal et al. 2009; Dobrovolskaia et al. 2009; Goppert and Muller 2005b; Kim et al. 2007). However, 'opsonins,' which are components of nanoparticle–protein corona, reported to act as a 'molecular signature', recognized by immune cells, determine the fate of the nanoparticle like kind of cell interaction, rout of internalization inside the cell, rate of clearance, distribution to different organs, etc. (Goppert and Muller 2005a; Kiwada et al. 1987; Muller and Heinemann 1989; Tyrrell et al. 1977). Interestingly, single-walled carbon nanotubes and albumin-coated silica

nanoparticles are reported to induce anti-inflammatory responses in macrophages, whereas another study reported that nanoparticle surface modified with nonionic surfactant (Pluronic F 127) to reduce the adsorption of albumin, inhibited anti-inflammatory response to the NPs (Dutta et al. 2007; Lynch and Dawson 2008). Additionally, the features of nanoparticles like rate of clearance and root of clearance from the body, organ deposition depend on nanoparticle–protein corona (Goppert and Muller 2005a; Tyrrell et al. 1977). It has been reported from various studies that all the biological responses to the NPs are possible due to surface area rather than mass (Brown et al. 2001; Donaldson et al. 2002; Donaldson et al. 1998; Muller and Heinemann 1989; Oberdorster et al. 1992). It is reported that, in some cases upon interaction with the nanoparticles, protein undergoes conformational changes resulting in loss of normal physiological function (Calzolai et al. 2010), resulting some unpredicted biological reactions including cytotoxicity (Lynch et al. 2006). Thus, the characteristic features of different nanoparticles inside the biological milieu vary depending on the physico-chemical characteristics of both nanoparticles and the biological entity.

Enzymes should retain their native structure and function for different applications in biological sciences. In this context, Asuri et al. have explored the structure, activity, and stability of different enzymes such as horseradish peroxidase, chicken egg white lysozymes, subtilisin Carlsberg by conjugating these enzymes with single-walled carbon nanotubes (SWNTs) (Asuri et al. 2007). The conjugation between different enzymes and SWNTs was covalent interaction. They have also characterized different enzymes upon conjugation with SWNTs using different biophysical techniques like circular dichroism and fluorescence spectroscopies. From extensive studies, they found that the enzymes retained their native structure and function upon attachment with SWNTs (Asuri et al. 2007). They also observed that different enzymes–SWNT conjugates are also stable in harsh chemical conditions like in guanidine hydrochloride (GdnHCl) solutions (Asuri et al. 2007). Hence, these enzyme–NP conjugates have attracted the scientists for different nanoparticle-mediated drug delivery. In another experiment, the native activity of two enzymes such as α-chymotrypsin and soybean peroxidase was observed upon adsorption onto single-walled carbon nanotubes (Wu et al. 2009). From the experiment, it was found that in case of the enzyme α-chymotrypsin, 1% of its native activity was retained, whereas in case of the enzyme soybean peroxidase, 30% of its native activity was retained (Wu et al. 2009). It is reported that the same nanoparticles help in the protein aggregation leading into amyloid fibril formation. For example, Aβ peptides are assembled to form fibrils in the presence of TiO_2 nanoparticles, since these nanoparticles accelerate nucleation process (Wu et al. 2008). De et al. have studied the refolding capacity of nanoparticles by choosing protein with positive residues on the surface (De and Rotello 2008). In their study, the protein was unfolded by thermal denaturation; hence, the hydrophobic inner cores were exposed to outside environment. The intermolecular interactions between the hydrophobic domains results in protein aggregation. They added malonic acid functionalized gold nanoparticles (AuDA) to these protein aggregates. Due to electrostatic interactions between nanoparticles having positive surface residues of

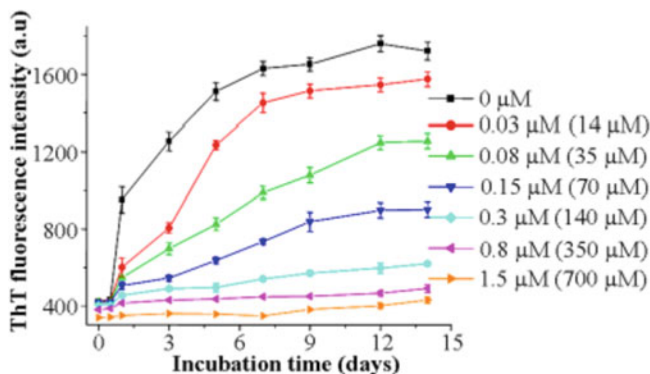

Fig. 8.1 Amyloid fibril kinetics of Aβ peptide in the absence and presence of histidine functionalized gold nanoparticles, as monitored by thioflavin T assay (Palmal et al. 2014)

proteins, nanoparticle–protein complex is formed. From zeta potential studies, they observed that high negative charge of nanoparticle–protein conjugates prevents the aggregation of the adsorbed protein (De and Rotello 2008). Shemetov et al. also observed the inhibition of Aβ-fibrillation in the presence of biocompatible nanogels (Shemetov et al. 2012).

Palmal et al. have also observed the effect of nanoparticles on Aβ-peptide fibrillation process. They incubated the peptide at different concentration of histidine-based functional groups gold nanoparticles at fibril-forming conditions. The concentration of Aβ-peptide was kept 25 μM and varied the concentration of nanoparticles from 0 to 1.5 μM. The amyloid aggregation kinetics was observed by thioflavin T (ThT) fluorescence assay (LEVINE-III H 1993). They found that the amyloid fibril formation is inhibited upon incubation with gold nanoparticle (AuNP) with histidine-based polymer coating, since ThT fluorescence intensity decreased with increase in AuNP fraction in the reaction solution (Fig. 8.1) (Palmal et al. 2014). They further observed the inhibition of amyloid fibril-like morphology when the protein is incubated with the nanoparticles using transmission electron microscope (TEM). Long amyloid fibrils were observed in the absence of nanoparticle, whereas no fibril-like morphology was visible when incubated with 0.8 μM of nanoparticles (Fig. 8.2) (Palmal et al. 2014).

8.3 Forces at NP–Protein Interface Affecting Adsorbed Protein Conformation

The different forces responsible for nanoparticle-biomolecular interaction are depicted in Fig. 8.3. Electrostatic interactions are most important forces those are charge specific. These forces attract or repel the charged protein molecules so that electrostatic double layer is formed. This charged double layer formed on nanoparticle surface creates the electrodynamic–Van der Waals interaction which may be

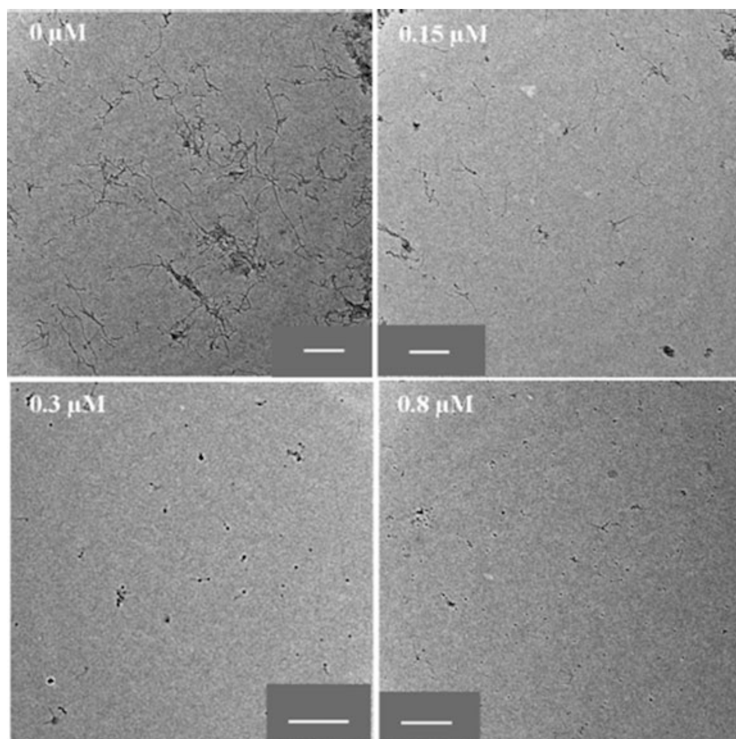

Fig. 8.2 Aβ-peptide fibrillation inhibition in the absence and presence of histidine functionalized gold nanoparticles using transmission electron microscopy (Palmal et al. 2014)

responsible for the structural and functional changes of adsorbed protein to some extent. The non-polar interactions with hydrophobic surface of protein lead to structural rearrangement of protein to a greater extent due to the exposure of inner regions of protein. This is because generally the hydrophobic domains of proteins are buried inside the protein. Though hydrophobic interactions are short range, they are responsible for the alteration of protein structure to a larger extent. All these forces described for nanoparticle–protein interaction are modulated by surface curvature of nanoparticles. It has also been studied that there is change in zeta (ζ)-potential, characteristic of charged surface with change in nanoparticles size (Shemetov et al. 2012).

It has also been studied that when the size of nanoparticle increases, there is decrease in isoelectric point of nanoparticles. It is assumed that decrease in isoelectric point is also a factor for nanoparticle interaction with biomolecules. Suttiponparnit et al. found that when particle size of TiO_2 increases from 6 to 104 nm, the isoelectric point of nanoparticle decreases from 6.0 to 3.8 (Suttiponparnit et al. 2011). These changes in isoelectric point may result in zeta potential change. Hence, the change in isoelectric point may also influence the interaction of nanoparticles with biomolecules (Shemetov et al. 2012).

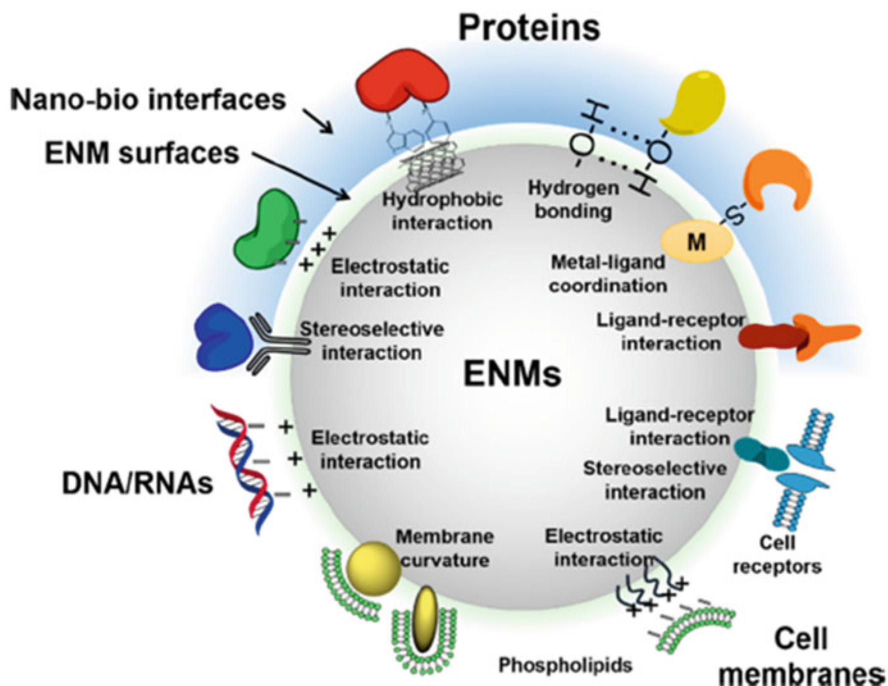

Fig. 8.3 Interactions at nanoparticle–biomolecule interface (Wang et al. 2019)

8.4 Factors Affecting Conformational Rearrangement of Protein at Nanoparticle Interface

When the biological molecules come in contact with nanoparticle interface, generally dynamic interacting components are observed, such as (1) the nanoparticle interface (the characteristics of nanoparticle interface depend on physico-chemical composition of nanoparticles), (2) the solid–liquid interface and (3) contact zone at solid–liquid interface with biological substance (Nel et al. 2009). Protein might undergo conformational rearrangement, when proteins are adsorbed onto the nanoparticle interface. However, many factors are there which are responsible for the interaction and extent of conformational changes in protein, out of which effect of size and concentrations of interfaces are discussed in succeeding headings.

8.4.1 Effects of Nanoparticle Size in Interaction with Protein

The size of nanomaterials affects the interaction pattern of protein/peptide with the nanoparticle at nanomaterial–protein interface. It has been reported that various proteins such as lysozyme, trypsin, horseradish peroxidase and catalase bind

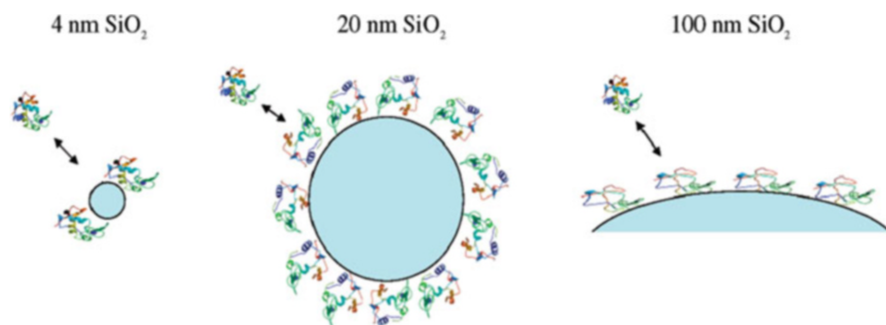

Fig. 8.4 Different sizes of silica nanoparticle affecting the interaction pattern of lysozyme with silica nanoparticle interface (Vertegel et al. 2004)

strongly to the SiO_2 nanoparticles (Vertegel et al. 2004). However, the study suggested that the partial loss of the protein structure influences significant loss of enzyme activity (Vertegel et al. 2004). As an extension of this work, Vertegel et al. have also found that the size of nanoparticle strongly influences interactions at the interface, studied by taking lysozyme and silica nanoparticle as model systems (Vertegel et al. 2004). It was found that the interaction was stronger in case of larger silica nanoparticles compared to smaller nanoparticles, leading to unfolding of lysozyme, hence resulted in insignificant lysozyme activity (Fig. 8.4) (Vertegel et al. 2004).

As shown in the figure, we can see that smaller silica nanoparticle has relatively higher surface curvature than larger silica nanoparticle. Hence, in case of protein interaction with smaller nanoparticle, the edge of the protein molecule will be at a greater distance from the NP surface, resulting in relatively weaker and non-cooperative interactions (both columbic and hydrophobic). Whereas, stronger and cooperative interactions are anticipated in case of larger nanoparticles due to the edge at closer distance. Hence, the extent of change in protein structure is relatively more significant when interacting with larger nanoparticles compared to smaller one. The loss of enzyme activity and α-helical content of lysozyme was also observed to greater extent upon its interaction with silica nanoparticles of larger size (Fei and Perrett 2009; Vertegel et al. 2004). Additionally, Shang et al. also observed similar results for silica nanoparticle upon interaction with RNaseA (Shang et al. 2007b).

8.4.2 Effect of Interface Concentration in Interaction with Protein

From extensive studies, it was reported that higher surface concentration of proteins helps in the interaction between protein molecules, because it helps in the adsorption of more proteins onto nanoparticle surface which makes a crowded environments. But, in lower concentration of protein, prominent interaction between nanoparticle and protein is observed (Fei and Perrett 2009). Wu and Narsimhan have studied the conformational changes of lysozyme upon interaction with silica nanoparticle of

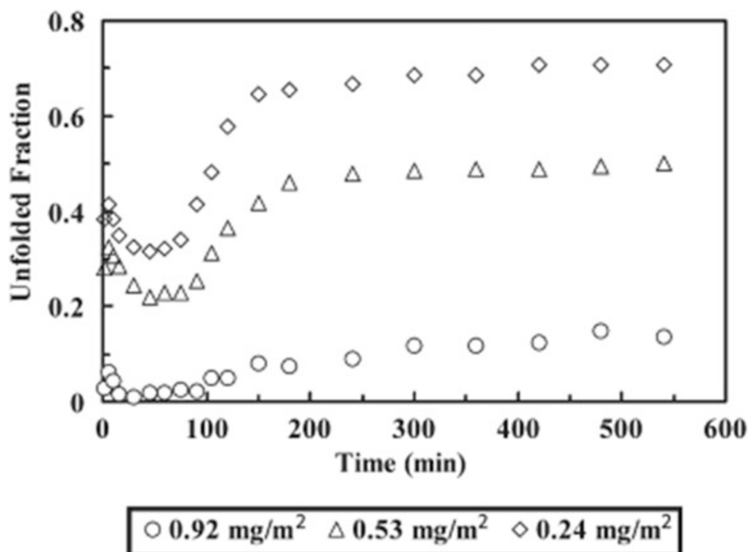

Fig. 8.5 Different surface concentration of silica nanoparticles affecting the unfolding kinetics of lysozyme at neutral pH (Wu and Narsimhan 2008)

different concentrations (Fig. 8.5) (Wu and Narsimhan 2008). At different concentrations of silica nanoparticle, the unfolded fractions of adsorbed lysozyme onto silica nanoparticle was calculated by the authors (Wu and Narsimhan 2008). From the study, it was observed that lysozyme was unfolded to a greater extent at low surface concentration in equilibrium state, which confirmed the existence of a high-energy barrier in a crowded environment (Wu and Narsimhan 2008). It is also reported that predominant interactions exists between proteins and the surface of nanoparticles at lower concentrations of protein molecules, leading to unfolding of protein, since free space is available and absence of energy barrier.

8.5 Potential Biological Implications of Nanoparticle–Protein Interactions

8.5.1 Possible Therapeutic Agents Against Protein Amyloidosis

The newly synthesized chain of amino acids fold into three-dimensional structures producing native structure. Native structure is functionally stable in local physiological conditions of protein. However, protein misfolding is a very common phenomenon of protein trafficking which occurs due to either mutations or change in local physiological chemical and physical conditions of proteins, or both. Some environmental factors, responsible for protein misfolding, are higher temperature, high or low pH, oxidative agents, elevated glucose, fatty acid level, etc. (Nelson and

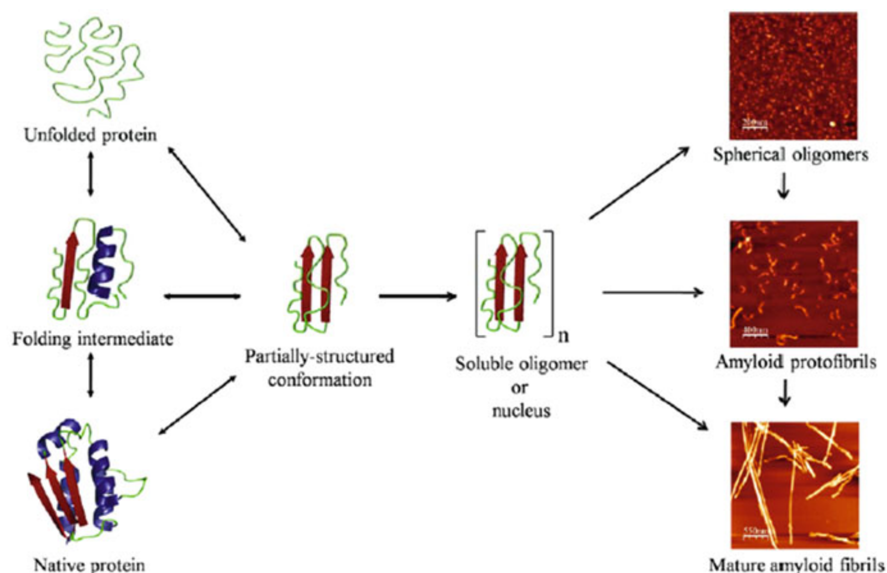

Fig. 8.6 Schematic representation of protein misfolding, aggregation. Atomic force microscopic images (right) showing amyloid fibrils (Kumar and Udgaonkar 2010)

Eisenberg 2006). After misfolding and failure of protein remodelling system, the misfolded protein kinetically or thermodynamically trapped in protein amyloid fibrillar structure (Fig. 8.6) (Kumar and Udgaonkar 2010). Protein amyloid fibrils are one specific form of protein aggregate which formed from self-assembly of misfolded proteins. These amyloid fibrils are different from other naturally occurring fibrils like collagen triple helix, keratin (Herczenik and Gebbink 2008). The most common features of amyloid fibrils are that they share a common core structure and cross β-sheet structure, and they bind fluorescent probes like Nile red, Congo red and thioflavin derivatives (Laidman et al. 2006).

When proteins are attached to planar surface, there are conformational changes in proteins. But nanoparticles are exceptional due to their high surface curvature, and less conformational changes occur to the protein. Studies have shown that some nanoparticles interact with proteins and enhance the aggregation propensity of proteins leading to the amyloid fibril formation. However, amyloid fibrils possess an alternative free energy minimum. These amyloid fibrils contain extended β-sheets aligned perpendicular to the elongation axis of fibrils. Several studies suggested that approximately 30 different proteins and peptides have been considered to be involved in the formation of amyloid fibrils inside the human body resulting in diseases (Chien et al. 2004; Chiti and Dobson 2006; Huff et al. 2003; Koo et al. 1999). Generally, in amyloid diseases, the soluble proteins self-assemble to form insoluble fibrils. It has been studied that some surfaces obtained by lipid bilayers, polysaccharides, native fibres (i.e. fibre like structure usually present in physiological condition to help cellular functions like vesicle trafficking, etc.), liquid–air,

liquid–solid, and liquid–liquid interfaces also help in either onset or prolongation of amyloid fibrillation (Knight and Miranker 2004; Myers et al. 2006; Yamaguchi et al. 2003). These studies confirmed that when proteins interact with different surfaces, physical or/and chemical adsorption of protein to the interface results in conformational rearrangement. Additionally, adsorption results in increased local concentration of the protein monomers. In case the conformational rearrangement results in exposure of hydrophobic patches or core, the increased local concentration of such monomers (conformationally compromised structure) on adsorption will result in self-assembly of the protein monomers into amyloid fibrils or other form of aggregates. In both the cases, protein monomer may not be available for usual physiological functions, hence onsets the disease.

Development of effective drugs against amyloidosis, which is strongly related to protein misfolding, has been a key issue from the last decade (Antosova et al. 2012). Worldwide research is going on to explore the novel therapeutics for the treatment of amyloidosis. The recent studies on nanoparticles have shown a novel possible approach for treating these incurable diseases (Kransnoslobodtsev et al. 2005). But unfortunately brief studies on several nanoparticles suggested that some nanoparticles like 70–200 nm copolymer particles, especially the thiol-linked nanoparticles, 16 nm hydrophilic polymer coated quantum dots, 16 nm cerium oxide nanoparticles, multiwall carbon nanotubes of 6 nm and TiO_2 nanoparticles have the potential to accelerate protein aggregation leading to fibril formation (Antosova et al. 2012). In contrast to the above findings, some nanoparticles have the potential to inhibit protein aggregation, so that these NPs can be used for the treatment of amyloidosis. Mrinmoy De et al. found that malonic acid–functionalized gold nanoparticles (AuDA) have that potential to refold the unfolded protein (De and Rotello 2008). Due to optical properties and density, gold can be easily observed in spectroscopic and microscopic techniques, and for its inert nature, it is a well-suited material for biological application (Antosova et al. 2012; Bellova et al. 2010). Bellova et al. studied the effect of magnetic (Fe_3O_4) nanoparticles on amyloid aggregation of lysozyme. They have studied this effect by thioflavin T fluorescence assay along with atomic force microscopy and found that magnetic nanoparticles interact with lysozyme amyloids in vitro. The interaction inhibited the amyloid aggregates by depolymerisation of the amyloid structure (Bellova et al. 2010). Apart from above, it has been found that fluorinated nanoparticles and hydrophobic teflon nanoparticles significantly inhibit Aβ amyloid polymerization (Rocha et al. 2008).

8.5.2 Antimicrobial Peptide Conformation at Nanoparticle Interface

Emergence of multidrug-resistant bacterial strains has become a serious threat to medical world (Arakha et al. 2016; Pal et al. 2019). Hence, different research groups are trying to develop novel antimicrobial agents against these strains. In this context, antimicrobial peptide (AMP) has attracted the interest of different research groups as

a broad spectrum of antibiotics (Pal et al. 2019). Due to the amphipathic nature of AMP, they efficiently target the membrane of microbes. However, the efficiency of these AMP has been compromised due to the emergence of these multidrug-resistant bacteria (Arakha et al. 2016; Pal et al. 2019). Hence, various research groups took the help of different nanoparticle-mediated approaches to enhance the efficacy of AMP. In this context, Arakha et al. have conjugated nisin, a widely used AMP in food industry with silver nanoparticles (AgNP). From different biophysical characterizations like UV-Vis, CD-spectroscopies and zeta potential analysis, they observed insignificant conformational rearrangement of nisin upon conjugation with silver nanoparticle (Arakha et al. 2016).

However, to evaluate the efficacy of nisin upon conjugation with AgNP, they have observed the antimicrobial activity of nisin at different ratio of AgNP-nisin conjugates (1:0.25, 1:0.5 and 1:1 w/w) against Gram-positive and Gram-negative bacteria like *Bacillus subtilis* and *Escherichia coli*, respectively, using growth kinetic analysis and colony-forming unit (CFU) measurements. From the experiments, they observed that, although nisin at nanomolar concentration shows insignificant antimicrobial activity, however upon conjugation with AgNP, the antimicrobial activity increased tenfolds higher compared to nisin (Fig. 8.7) (Arakha et al. 2016). Nisin adsorption onto AgNP enhanced the effective local concentration of nisin interaction with bacterial membrane surface, which is needed for membrane pore formation. Nisinase was sterically hindered to act upon the AgNP-adsorbed nisin. Hence, the complex was effective against the bacteria which have evolved the nisinase-based resistance against the nisin. In another study, Pal et al. have conjugated a potent AMP such as Andersonin-Y1 with AgNP and observed that the resultant conjugate exhibits enhanced antimicrobial activity by tenfolds higher against multidrug-resistant strains (Pal et al. 2019). From MD simulations, they have concluded that bacterial cell death was due to pore formation in the membrane, which is due to hydrophobic collapse mechanism. Hence, the above studies confirmed that the AMP can be a potential antimicrobial drug upon conjugation with nanoparticles against multidrug-resistant bacteria.

8.5.3 Use of Enzyme Nanoparticles as Biosensors

It has been reported that enzyme molecule can aggregate themselves to a nanoscale size and forms nanoparticle-like structure. However, the interaction playing key role here is the interaction between the enzymes forming nanoparticles. These enzyme nanoparticles are used in preparation of different nanobiosensors (Fig. 8.8). For example, nanoparticles from lipase, glycerol-3-phosphate oxidase, glycerol kinase are prepared separately, and then immobilized on an gold (Au) electrode for the preparation of triglyceride bionanosensor (Chen et al. 2017; Pundir and Aggarwal 2017). Additionally, Narwal et al. also immobilized the above three enzyme nanoparticles on pencil graphite electrode (Narwal and Pundir 2017). These enzyme nanoparticles are widely used in the construction of different biosensors for detection of molecules like triglyceride and uric acid (Chen et al. 2017). These enzyme

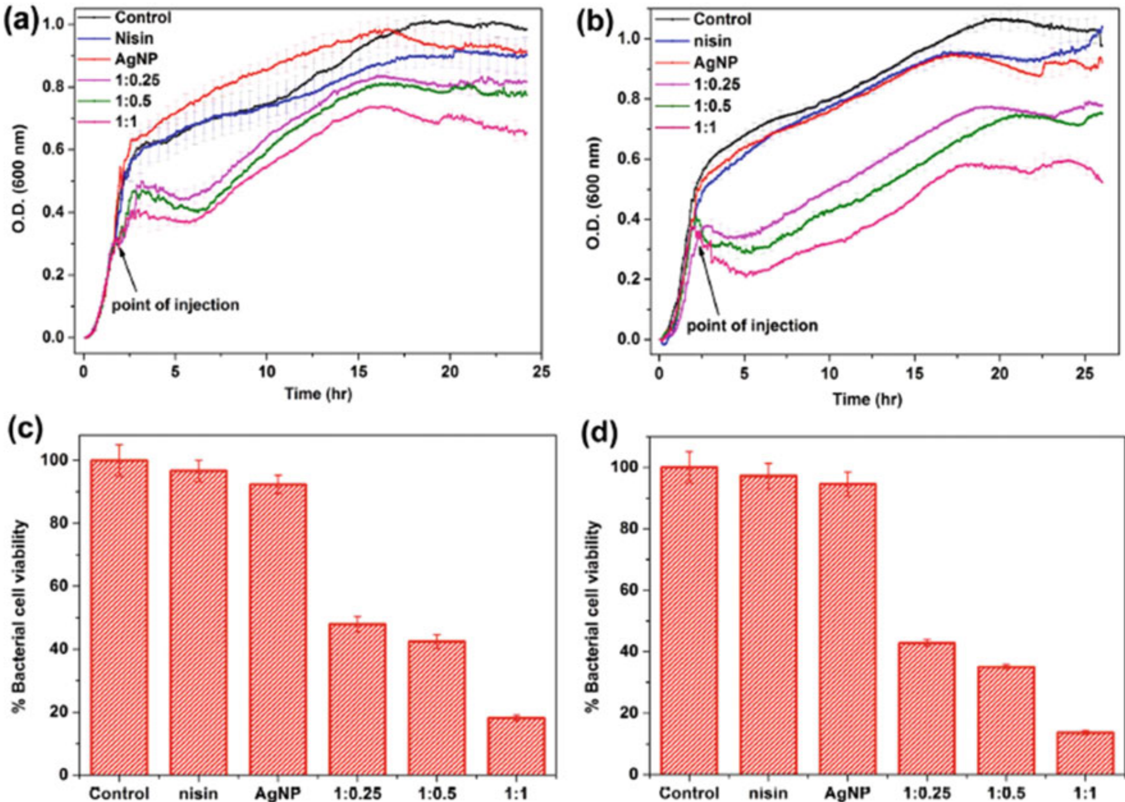

Fig. 8.7 Evaluation of antimicrobial activity of AgNP, nisin, and deferent AgNP-nisin conjugates by growth kinetics study of *B. subtilis* (**a**) and *E. coli* (**b**). Measurement of colony-forming units (CFU) for *B. subtilis* (**c**) and *E. coli* (**d**) in the presence of AgNP, nisin and deferent concentrations of AgNP-nisin conjugates (Arakha et al. 2016)

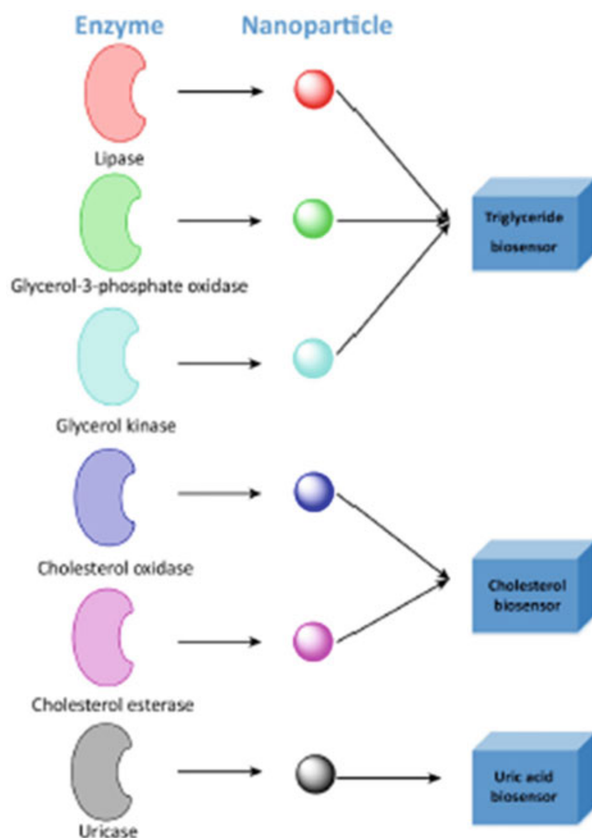

Fig. 8.8 Examples of different enzyme nanoparticles for construction of biosensors

molecules are immobilized onto different electrodes to improve the performance of biosensor (Chen et al. 2017).

8.6 Conclusion

Nanoparticle–protein interaction is a promising field for current and future research. Deep understanding of conformational rearrangement of protein upon interaction with nanoparticles can help in various ways like in treating protein misfolding diseases as well as in adopting different therapeutic approaches using nanoparticles. In this chapter, we discussed about the studies done on nanoparticle–protein interaction and the effect of nanoparticles on protein/peptide conformation. The chapter also discussed different biological applications of nanoparticle–protein interaction. Generally, most of the studies done so far were in vitro studies. Further in vivo studies are needed for better confirmation about the possible therapeutic roles of nanoparticle in protein misfolding diseases.

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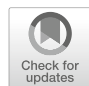

Enzyme-Nanoparticle Corona: A Novel Approach, Their Plausible Applications and Challenges

9

Mainsh Paul, Niteesh Pandey, Gireesh Shroti, Preeti Tomar, Hrudayanath Thatoi, Debalina Bhattacharya, Samdra Prasad Banik, Debashish Ghosh, and Saugata Hazra

Abstract

Protein coronas incorporate with nanoparticles (NPs) are now becoming a new trend in research and can introduce novel applications in various fields and majorly used in biomedical aspect. The unique features presented by protein coronas can be exploited in the design of the nanomaterial, rather than combat their adsorption. Uncontrolled protein coronation may lead to cytotoxicity, a reduced blood circulation half-life, and nonspecific targeting to a diseased cell. However, a comprehensive understanding and design of suitable nanomaterials with varied functional proteins can allow selective protein coronation, which can help to tailor their therapeutic properties for nano-drug delivery vehicles. Protein corona is a complex structure and its uniqueness varies with different nanomaterials and nanoparticles. Upon contact with biological media,

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biomolecules adsorb onto a nanoparticle's surface forming a layer mainly composed of proteins, which is called protein corona. Formation of protein-NP corona is influenced by many parameters such as the nanoparticle's physicochemical properties and biological environmental factors. This dynamic structure constitutes the new interface with biological systems and consequently has a deep impact on the nanoparticle's biological fate and response. In summary, it can be stated that novel investigations about protein-NP corona will assist to develop potential therapeutic against life-threatening diseases and other applications.

Keywords

Nanotechnology · Nanoparticles · Enzyme-nanoparticle corona · Drug delivery · Gene therapy · Enzyme immobilization · Biosensor

9.1 Introduction

Protein corona has different applications in biomedical, biotechnology, and industrial sectors. Protein corona is formed by the interaction between different biomolecules (e.g., proteins, DNA) with the surface of nanomaterials (Mahmoudi et al. 2011a, b; Monopoli et al. 2012). The mechanism of adsorption by protein coronas should be well characterized in order to accurate interpretation of the interactions between nanoparticles and cells (Monopoli et al. 2013). There are different biomarkers present which accumulate over nanoparticles after their entry within biological fluids like blood plasma, serum etc. (Maiolo et al. 2014). Proteins that have high binding affinity for nanoparticles are called hard corona (HC) proteins whereas the proteins with low affinity toward nanoparticles are called soft corona (SC) proteins. HC-nanoparticles composites are comparatively more stable with a long interaction time than the dynamic SCs which have shorter interaction time (Maiolo et al. 2014; Hadjidemetriou et al. 2015). Protein corona has been reported to protect cells from the scavenging attacks of phagocytotic cells while the bare nanoparticle surface with no protein bound state is more prone to engulfment by enzymatic lysis and eliminated through the process of phagocytosis (Wang et al. 2013). Enzyme-NP corona has now been mostly used in vector-mediated drug delivery in biomedical companies. Gene delivery using enzyme-NP corona has also become an interesting field of research in the purpose of treating genetic disorder and also traits development. Other than these, enzyme-NP corona has significant uses in immunoassay, cytotoxicity assay and many other fields.

9.1.1 Enzyme Nanotechnology

Enzyme nanotechnology is a new and fast developing branch of science which enables the production of desirable nanomaterials that have various applications in the field of medical science. One of the advanced uses of enzyme nanotechnology is

nanozyme which is an artificial enzyme-based nanomaterial. Due to efficient designing and catalytic site flexibility of nanozyme it becomes better biocatalyst. Nanozyme mainly work on active sites mimicking of traditional enzymes or copying enzymatic catalytic activity with high stability and low cost. Through deep understandings of catalytic site of nanozymes will help to develop more efficient and effective nano-catalyst. Incorporation of nanotechnology in protein engineering makes nanozymes more applicable to various sectors like, biomedical, industrial, and agricultural. Moreover, use of nanoparticles provides a good platform to do modifications in nanosystem according to need and requirements of nanozymes. Recent advancements in nano-science not just allow modifications nanozymes but also gives platform to develop tailor-made multi-substrate nanozyme systems (Jiang et al. 2019). Nanoparticles bound with enzyme mediate different type of reaction catalysis. Enzymes like α-amylase, β-galactosidase, cellulase, lipase, etc. have reported to use in nanotechnology for the construction of enzyme-NP corona. Recent development in nanotechnology especially in the field of nanoscaffolds has provided a wealth of success. These newly evolved nanoscaffolds in combination with NPs have potential uses in enzyme immobilization which can be applied in biotechnology, immunosensing, biomedical, and industrial areas.

9.1.2 Structure and Composition of Corona

Protein corona mainly composed of two sections: Hard corona and soft corona.

9.1.2.1 Hard Corona and Soft Corona

Cedervall et al. (2007a, b) first ever coined the term Protein "coronas," which is a complex structure composed of soft and hard layers, known as the soft and hard corona, respectively. In hard corona, nanoparticles remain tightly bound with protein whereas in soft corona nanoparticles remain loosely bound with protein complex (Fig. 9.1). Therefore, the absolute binding free energy between nanoparticle and enzyme during formation of hard corona is high compared to that of in soft corona. Due to strong interaction between the nanoparticle and enzyme in hard corona, first-order rate constant for the dissociation energy is high while in case of soft corona this energy value is reported to be low.

In hard corona, formation of proteins interacts directly with nanomaterials but in case of soft corona, protein–protein interaction formation takes place at first and then this protein–protein network going to link with nanomaterials. The protein interacting with nanomaterials remain stable on nanoparticle surface and able to influence the functional response. But in case of soft corona, proteins assembled on nanoparticle surface does not have significant impact on functional response. SC proteins shown to interact with NPs secondarily because HC proteins present directly to the core NP surface (Walczyk et al. 2010). Cedervall et al. (2007a, b) reported that the SC components have a relatively shorter interaction time with NPs while HC components bind to NPs for longer time. This observation also suggested that due to the long interaction time, HC components have more affinity with NP

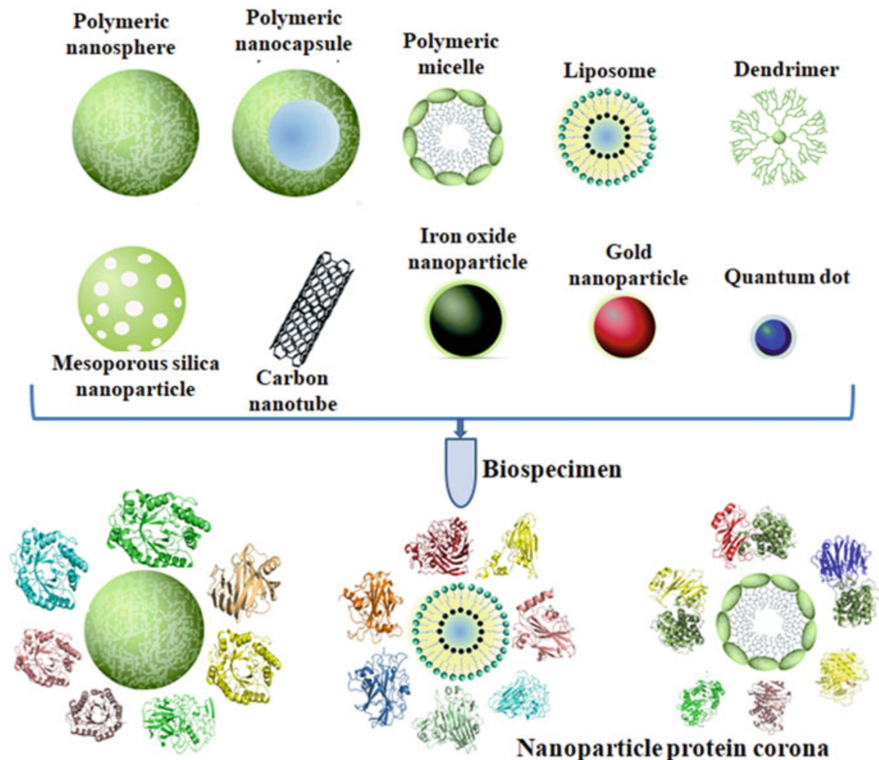

Fig. 9.1 Demonstration of stepwise development of a nanoparticle-protein corona

compared to SC components. Walkey and Chan (2012) suggested in their study that the HC proteins are more significant than the SC proteins in regulating the activity of the protein-based nanomaterials because the HC proteins have longer interaction time compared to SC proteins.

9.2 Interaction of Nanoparticles with Blood

After systemic administration of nanoparticle in animal body, it is first exposed to blood. In the flow of bloodstream, nanoparticles are shown to interact with proteins like serum albumin or apolipoproteins to form nanoparticle–protein corona. In bloodstream, intrusion of a protein-NP corona can cause the following phenomena like: hemolysis, complement system activation, adsorption, aggregation, and internalization (Fig. 9.2). After interaction with different proteins in blood, the nanoparticles gain a new identity with novel physiochemical properties which then exhibit its activity. In a study, iron oxide NPs was incubated in fetal bovine serum (FBS) to investigate the impact of SC and it was observed that the corona consisted of mostly complement proteins, antithrombin, and α-antiproteinase (Sakulkhu et al.

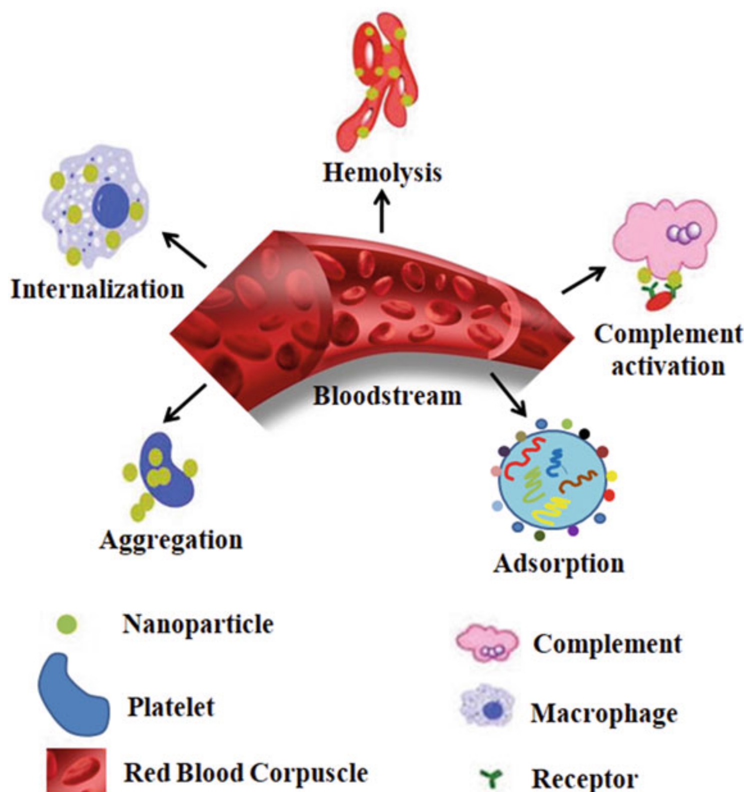

Fig. 9.2 Interaction of nanoparticles with blood and subsequent physiological changes

2014a, b). In an another study conducted by Bonvin et al. (2017), it was interestingly found that the complement proteins were mainly remains in the HC, rather than the SC as in the previous study (Sakulkhu et al. 2014a, b). A possible explanation from this comparative observation as demonstrated by Sakulkhu et al. is that the surface chemistry of NP differed with variable amount of polyvinyl alcohol. There are some proteins like: apolipoproteins, serum albumin, fibrinogen, and immunoglobulins which are frequently used in the formation of enzyme-NP corona. NP are also of different types, such as metalloids (Izak-Nau et al. 2013), liposomes (Pozzi et al. 2014), and polymers (O'Connell et al. 2015). A class of proteins known as opsonin shown to trigger the clearance of NPs from blood through reticuloendotheilal system and reduces their circulation time in blood (García-Álvarez et al. 2018). Chen et al. in their study illustrated enzyme-NP corona-mediated rapid opsonization incubated in both human plasma and serum. The opsonization was shown to be activated by the complement component (C3) (Chen et al. 2017). Nanoparticles are often reported to get aggregated on platelets in bloodstream whether some nanoparticles may also get internalized under a macrophage. These phenomena are likely to occur for the

maintenance of nanoparticle concentration in blood during or after its activity (Radomski et al. 2005).

9.3 Interactions of Enzymes with Nanoparticles or Nanomaterials

Interaction of nanoparticles with enzymes is the basis in the formation of enzyme-NP corona. Proper formation of interactions is required for the accurate dynamism of enzyme-NP corona and hence its target-specific cellular uptake. There are varieties of attractive and repulsive forces take place between enzyme and nanoparticles or nanomaterials. These interactions include: electrostatic interactions, hydrophobic interactions, hydrodynamic interactions, electrodynamic interactions, solvent interactions, steric interactions, π–π stacking, salt bridge, and polymer bridging interactions. Other than these forces there are also forces like: molecular force, magnetic force, and entropic force that may be present in enzyme-NP corona (Fig. 9.3).

Interaction between enzymes and nanoparticles depends on some physicochemical characteristics of nanoparticles such as: shape, size, surface, material, and the pH

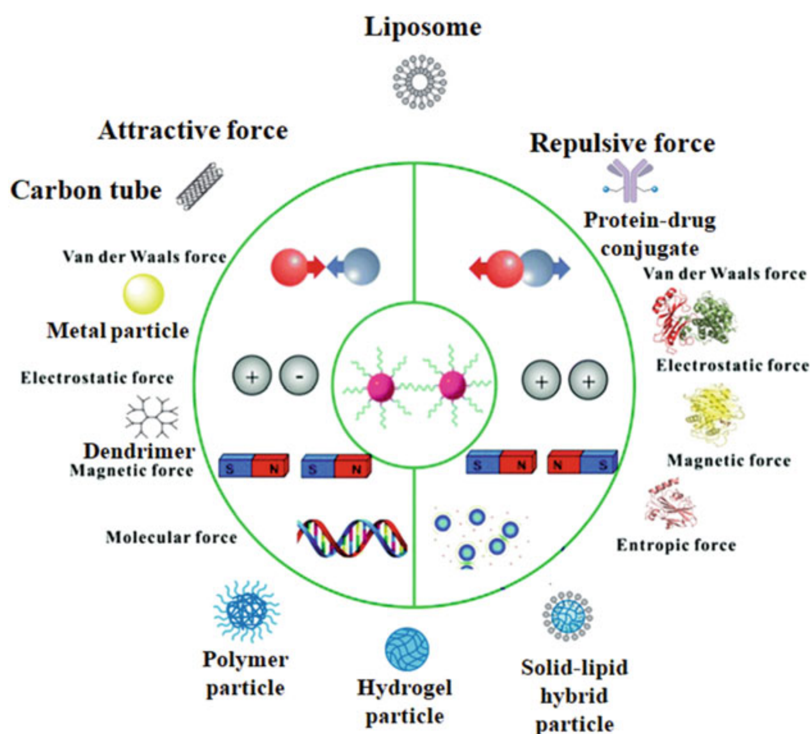

Fig. 9.3 Different forces in the formation of enzyme-NP corona

and temperature of the environment. Favorable energetic during the formation of these interactions between enzyme and NP are indispensible for the stabilization of protein corona and its errorless activity. High electrostatic energy on the surface of nanoparticle is shown to have higher binding affinity with enzymes. Furthermore, the NP surface can induce different conformational changes in enzymes acquired from the variation in the interaction energy. The enzyme–NP interaction is also varied upon the characteristics of organic and inorganic biopolymers. There are different types of biopolymers which include: carbon nanotubes, dendrimer, protein–drug conjugate, polymer particle, liposome, solid–lipid hybrid particle, hydrogel particle, metal particle, etc (Hühn et al. 2013). DNA as a biopolymer can also interact with nanoparticle with the molecular force. In-depth understanding of such forces and interactions is needed toward the construction of biocompatible enzyme-NP corona.

9.3.1 Hydrodynamic Interactions

Hydrodynamic interactions between the enzyme and nanoparticle are regulated by the Brownian diffusion between interacting interfaces of two molecules. This interaction increases with the collision between by nanoparticles by convection of dragging, shearing, and lifting of nanomaterials. Range of this interaction varies between 10^2 and 10^6 nm. For example, this type of interaction is found in the enzyme alkaline phosphatase bound to silver NP.

9.3.2 Electrodynamic Interactions

This interaction mainly includes van der walls (VDW) interactions which arise from the interacting molecules and surrounding media. This interaction is significantly found in aqueous media while it is very uncommon in biological media. Range of this interaction varies between 1 and 100 nm. This type of interaction causes allosteric binding of the enzymes on the surface of nanoparticle.

9.3.3 Electrostatic Interactions

Electrostatic interaction is mainly formed due to Coulombic forces between two materials. During enzyme–NP interactions, charged interfaces come in close contact through Coulombic forces causing the formation of an electrostatic layer. Overlapping electrostatic layers formed during enzyme–nanoparticle interactions are generally repulsive, but are able to attract oppositely charged materials. Range of this interaction can also be varied between 1 and 100 nm (Irle et al. 2003; Hong et al. 2004).

9.3.4 Solvent Interactions

During the formation of enzyme–NP complex, solvent interaction mainly occurs between the solvent molecules with lyophilic and lyophobic materials. Lyophilic materials shown to interact favorably with solvent molecules while lyophobic one interacts unfavorably. These interactions range between 1 and 10 nm.

9.3.5 Hydrophobic Interactions

This is a strong interaction formed between nanoparticles and enzymes with a range of 0–10 nm. These interactions are found in case of partial specificity during the interaction between nanoparticle and enzymes. This interaction is dependent upon the hydrophobic surface of the interacting molecules (Strano et al. 2003; Erlanger et al. 2001).

9.3.6 π–π Stacking

Like the hydrophobic interactions, it is also a strong interaction that ranges between 0 and 5 nm. Due to π–π stacking, nanoparticle and enzyme binds specifically with each other. This interaction is greatly affected by the aromatic ring orientation in the interacting molecules (Lin et al. 2004; Li et al. 2006).

9.3.7 Salt Bridge

Salt bridge is one of the strong interaction formed between nanoparticles and enzymes with a range of <1 nm. This interaction also provides specificity during the interactions between nanoparticles and enzymes as in found in π–π stacking. Formation of salt bridge during nanoparticle–protein interaction is dependent upon multiple factors like: the partial charge of the interacting atoms present in the molecules, dihedral angles of two interacting atoms in protein residues, etc.

9.3.8 Steric Interactions

This type of interaction varies between the ranges of 1 and 100 nm. This interaction type is mostly found during the irreversible enzyme-NP interactions where enzymatic inhibition takes place. Steric interactions can be design in the formation of enzyme-NP corona to avoid mistargetting during the cellular uptake of protein based nanomaterial.

9.3.9 Polymer Bridging Interactions

This interaction generally promotes aggregation or deposition of nanoparticles upon the interacting surface particularly when charged functional group is carboxylic acid. It has been reported that in the presence of calcium ions, nanoparticles get dispersed in aqueous media due to this interaction. Polymer bridging is shown to take place in tubulin protein during its interaction with TiO_2 NP. Due to this interaction, conformational changes in tubulin structure has been reported which cause decrease in protein polymerization.

9.3.10 Reversible and Irreversible Interactions Between Protein and NP

Proteins can bind with NP both in reversible or irreversible mode. Both the interaction mode can alter protein conformation and behavior. Reversible interaction between protein and NP allow retaining protein structure and also permitting protein to interchange between various structural conformations. Irreversible interaction of protein with NP presents a greater challenge in understanding the functionality of nanomaterials. Retaining of protein's 3D structure and correct folds are important for its functioning.

In a previous study, negatively charged anionic NPs were used to inhibit the enzymatic activity of proteins, such as α-chymotrypsin (You et al. 2006). This type of nanoparticle was used to build a model for elimination of unwanted protein traffic in corona formation and is efficiently used in drug or gene delivery system in conjugation with the desired corona that have to incorporate within the target cell.

Structural denaturation of protein was reported due to the interaction between the nonpolar interior core of the NP with the hydrophobic residues in α-chymotrypsin. Application of the enzyme-NP conjugates requires retention of the protein activity and is designated as a factor of most priority. The surface of NPs can be frequently engineered to prevent the structural denaturation of proteins. It has been shown that the introduction of polyethylene glycol (PEG) on the surface of NP drastically reduces the denaturation of α-chymotrypsin (You et al. 2005). This approach is used in selective NP-enzyme corona formation.

Structure of interacting protein is a crucial determining factor for the level of stabilization of enzyme-NP corona. It has been shown that if an interacting protein has a rigid structure and its active site remains buried deeply inside the core of the protein structure, then the stability of enzyme-NP corona is unaffected. If the interacting protein has flexible structure and its active still buried deeply in the core region, then the stability of enzyme-NP corona can be affected but it does not affect the enzyme activity. If the interacting protein is flexible and its active site orient toward surface then both the stability of enzyme-NP corona and the enzyme activity can be affected. Understanding these comparative differences and the structure–activity corelationship is significant for the assessment of interactions occurs at bio-nano interface during the enzyme-NP corona formation (Shcharbin et al. 2015).

9.4 Physicochemical and Dynamic Factors in Corona Formation

Physicochemical characteristics of NPs such as shape, size, surface charge, hydrophobicity, presence of specific functional groups, pH, and temperature are the important variables that have significant impact on protein-NP corona formation (Auría-Soro et al. 2019). Hu et al. (2014) in their study explored the effect of nanoparticle size on the protein corona formation by incubating iron oxide particles of different sizes about 30, 200, and 400 nm in human plasma. The result obtained from this experiment showed that the 30 nm particles exclusively interact with the proteins that regulate cell cycle, whereas the protein bound with 200 nm particles responsible for different physiological processes. The 400 nm NPs shown to had no significant functional associations. Nanoparticle-protein corona formation also depends on the function of pH and temperature. Gorshkov et al. (2019) have reported that the binding affinity of human plasma proteins on the surface of silver nanoparticles does not alter with the variation of pH and temperature. In this context, Mahmoudi et al. have studied the effect of temperature fluctuation on the PCs formation and its related composition on cellular uptake (Mahmoudi et al. 2013). They incubated dextran-coated FeO_x NPs with different surface charges with FBS at various temperatures and found that cellular uptake of NPs is altered by the temperature but not the composition of PC.

Biological dynamics has major impact on enzyme-NP corona formation. One of the aspect in biological dynamic is known as "Vroman effect," first ever studied by Vroman (1962). The Vroman effect describes the phenomenon that the arrangement of the proteins in a corona may be changed in a regular time interval due to the positional exchange of those proteins bound to the surface of nanoparticle (Zhdanov and Cho 2016). Soft corona shown to have a higher degree of positional transitions between proteins bound to its surface. This type of biological dynamics of proteins have been reported in the study by Tenzer et al. (2013) where enzymes were employed to bind with silica-based nanoparticles in human plasma. It was found that the degree of adsorption of the members from same protein family onto NPs surface was majorly relies on incubation time and nanoparticle charge (Tenzer et al. 2013).

Further, in an in vivo study conducted by Chen et al. (2017), significantly complex dynamism of complement C3 binding protein were observed when the protein interacted with iron oxide nanowires during the adsorption and de-adsorption on NPs surface (Jansch et al. 2012). Moreover, it has been reported in a study that the order of the binding of plasma proteins was also depend on the surface of the nanoparticles (Ge et al. 2011) (Fig. 9.4).

During the adsorption of enzyme-NP corona in plasma, the Vroman effect occurs through two stages. These are "early" and "late" stages. In the early stage, rapid adsorption of albumin, IgG, and fibrinogen takes place followed by the adsorption of apolipoprotein and coagulation factors in the second step (Goppert and Muller 2005b). All the nanomaterials did not show to have the early stage of Vroman effect. The late stage of Vroman effect was shown to be occurred in case of those proteins

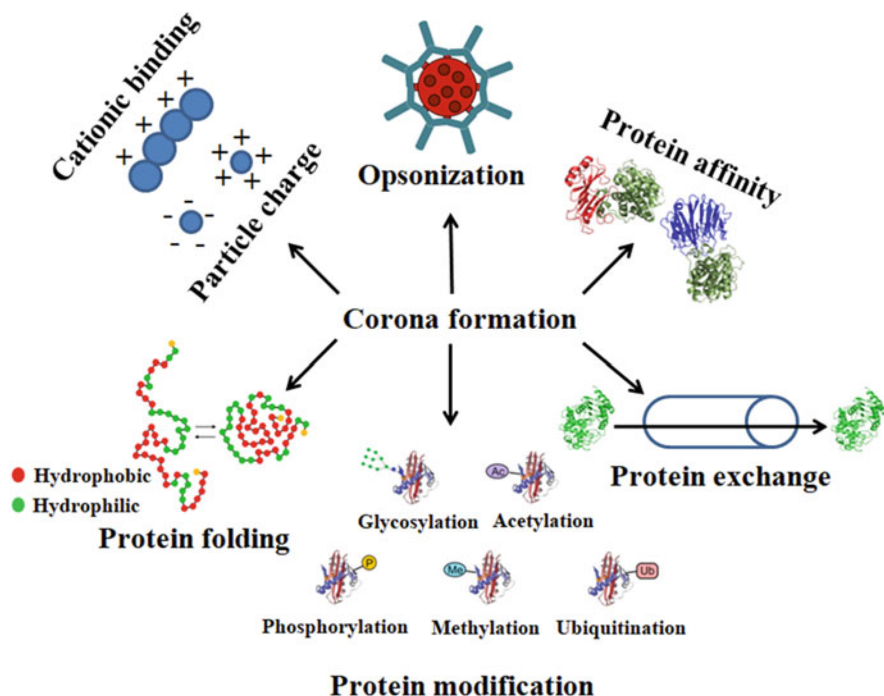

Fig. 9.4 Several factors affecting the formation of enzyme-NP corona

which have moderate affinity for the nanoparticle. Another important factor for protein corona formation is the amount of plasma proteins available to interact with the surface of nanoparticles.

In an investigation, it was shown a different affinity between the protein and NP surface when silica NPs was incubated with a range of plasma protein concentration of 3%, 20%, and 80% (Gräfe et al. 2016; Monopoli et al. 2011). Result from the study depicted that the possibility of enzyme-NP corona formation is greatly increased and stabilized with the increase in protein concentrations. For the formation and stabilization of enzyme-NP corona, fundamental thermodynamics should be maintained at the interface of interacting enzyme and nanoparticle.

9.4.1 Early Stage

In the early stage, albumin, IgG, and fibrinogen shown to be get rapidly adsorbed upon the administration of nanomaterials within the biological environment. During this stage, exposure of nanoparticles in blood causes the formation of a layer of serum albumin on the surface of most of the nanomaterials as the concentration of serum albumin in blood plasma is very high. This layer of serum albumin gets replace with the high affinity protein over the time (Lundqvist et al. 2011).

9.4.2 Late Stage

The longer incubation of protein-NP corona in biological fluids causes the replacement of fibrinogen and apolipoproteins (Goppert and Muller 2005a). A change in the protein adsorption patterns is also reported with time which could result in the change in distribution pattern of nanoparticles in different organ.

Protein corona formation also depends upon some other physicochemical and environmental parameters like charge density, hydrophobicity, and curvature of NP, and also protein concentration present in the biological fluid. Higher charge density on the surface of NP was shown to increase the thickness of corona. Charged nanoparticles has comparatively higher rate of opsonization. High charge density of NP can increase protein conformational change. A higher hydrophobicity of NP also cause increase in corona thickness, opsonization rate, and protein conformational change. Higher curvature in NP surface reported to increase corona thickness as a more number of proteins can accumulates in this type of NP surface. Whether, a higher curvature in NP surface shown to decrease the conformational change in bound protein. Higher protein concentration in biological medium causes the thickness of protein-NP corona (Aggarwal et al. 2009; Walkey and Chan 2012).

9.4.3 Protein Conformation

Protein conformation is reversible and refers to three dimensional shapes that can be arranged with its different structural characteristics. Conformations of a protein can always get interchange between one form to another depending upon surrounding environmental factors and its activity. During the interaction with other biological or non-biological molecules, proteins structures may rearrange continuously through a dynamical process. When the proteins bind with NP to form protein corona, protein may restructure their conformation to adapt to fit on the NPs surface and surrounding biological fluids. This event is called conformational change in which the protein adjusts itself with its exterior medium.

A small modification of the interaction between NP and enzyme can have a greater impact on protein structures which further induce a significant change in the pharmacological activities of enzyme-NP interaction in the body and therefore a notable change in biological response (Lynch and Dawson 2008).

There has a correlation between NP sizes with protein structural change. If the NP is bigger than proteins in size, then the interacting proteins can manage to stretch themselves for adapt in the NP surface. In such case, significant structural modification for the bound proteins can be noted. But in case of smaller NPs less structural changes is found for the interacting proteins because the bound protein can't get enough space to extend their structure for further rearrangement (Koegler et al. 2012). Changes in the secondary structure of protein were shown to be strongly influenced by the surface charge on NPs. For example, it has been reported that, gold NPs with identical properties, but different surface charges, presented similar adsorbed capacity for bovine serum albumin (BSA). However, the positively

charged NPs showed much higher affinity for the enzymes and a faster cellular uptake than the negative one. This result suggested that the overall structural modification in the protein BSA is caused by the NP surface charge. In positively charged NPs, bound BSA undergoes more conformational changes than in the case of negatively charged NP surface, which facilitate the ease in the internalization of enzyme-NP corona inside the target cell. It has been shown that, the adsorbed BSA by negatively charged polystyrene NPs remain in its native structure, makes its way toward the scavenger receptors, compared with the BSA that get conformational modification after binding with positively charged NPs.

The adsorbed BSA on negatively charged anionic polystyrene NPs could inhibit cellular binding of other BSA molecules that might have chance to be go inside the target cell after interacting with positively charged NPs. Therefore, the cellular system make those BSA bound with negatively charged NPs destroyed by scavenger molecules (Fleischer and Payne 2014).

9.5 Characterization of Different Protein–Corona Related Parameters

Protein corona formation is characterized by different parameters and their variations. Assembly patterns of polypeptides and the environmental factors are also some major parameters that play significant role in the formation of nanomaterial based protein corona. Polypeptides assemble to form proteins and provide them a specific charge at the physiological condition that may vary for different suspension media. Formation of protein corona around the materials exposed to biological fluid (physiological condition) is a universal phenomenon (Lynch et al. 2007). NPs-corona formation is dependent on NPs in terms of its surface properties like charge, shape, morphology, prevention from aggregation, hydro dynamicity, etc.

Formation of protein corona may get affected due to variation in the physico-chemical properties of NPs viz. shape, size, surface morphology, pH, and temperature. Protein corona formation can be affected by various other parameters like adsorption rate, isothermal characteristics, homogeneity of NPs, etc.

Protein corona formation is a dynamic process and it can be viewed in terms of:

9.5.1 Physicochemical Properties of NPs

Physicochemical properties like size, charge, shape, morphology, heterogeneity of NPs may vary from one NPs to another depending on method of synthesis and conditions applied for the synthesis, which can affect the formation of protein corona around the NPs (Saptarshi et al. 2013; Boselli et al. 2017; Heyes et al. 2004; Vertegel et al. 2004; Zarschler et al. 2016; Lundqvist et al. 2017).

9.5.2 Nature of Protein

It is the scenery of protein that plays a major role in the formation of protein corona in terms of association/dissociation constant of proteins, their stability and form, etc. Specific binding efficiency and binding energy of proteins play a vital role toward the formation of protein corona (Cedervall et al. 2007a, b; Lundqvist et al. 2004). Amount of proteins available in the surrounding medium of NPs make significant changes in the protein corona formation as shown in Fig. 9.2. Ultimately, the nature of proteins present in the corona determines the distribution of NPs in the biological fluid and the fate of NPs which is further specific to different cellular types. Monopoli et al. (2011) found that while increasing the concentration of plasma from 3 to 80%, there is an increase in protein adsorption on NPs.

9.5.3 Precoating on NPs

Precoating on NPs is needed either due to travelling through different biological environments in an in vivo condition. NPs are coated with some biomolecules such as proteins, lipids, carbohydrates, etc.

Different types of precoating can change the effectiveness of NPs in terms of formation of protein corona e.g., pulmonary surfactant coating has been reported to alter the protein adsorption on multiwalled carbon nanotubes (MWCNT) (Ogawara et al. 2004; Rojas et al. 2016; Mirshafiee et al. 2016). The probable reason of variable effectiveness of NPs could be the NPs charge and other physiological conditions.

9.6 Applications of Enzyme-NP Corona

Nanoparticles are used in many aspects like disease diagnostics, development of biomedicine, gene therapy, cytotoxicity assay, immunotoxicity assays, enzyme immobilization, etc. (Fig. 9.5).

9.6.1 Drug Delivery

Protein corona-nanoparticle cellular interphases are also effectively used in drug delivery. Still many of the properties of nanoparticles are under investigation and it is strongly believed that the subsequent environmental and physiological conditions for protein corona formation have major impact on drug development and further downstream in vivo applications of the developed drug (Behzadi et al. 2014; Cifuentes-Rius et al. 2013; Hazra et al. 2009; Kah et al. 2012; Sabini et al. 2008). Cifuentes-Rius et al. (2013) showed that the rate of drug delivery can be altered by modifying the corona composition around nanoparticles. They proposed from this study that the buffer strength in media and the concentration of human serum and cetyltrimethylammonium bromide are the major factors in the formation of enzyme-

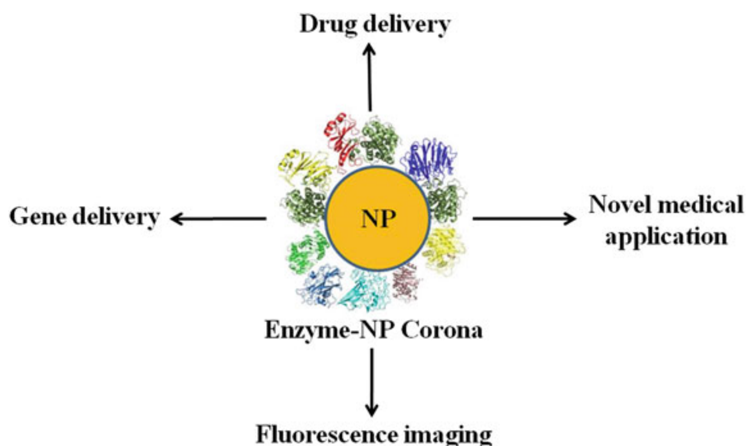

Fig. 9.5 Applications of enzyme-NP corona

corona and successful drug delivery as well. Kah et al. (2012) showed in their study that the cetyl trimethyl ammonium bromide coated coronas formed by serum proteins on gold nano-rods exhibit a ~5 to ten fold greater success rate as therapeutics. Drug carrying capacity is an important factor of enzyme-NP corona during the drug delivery. This defines the amounts of drug molecules that can be successfully transferred inside the target cell by the designed corona without forming any toxic substances. It has been shown that the drug carrying capacity of protein corona varies with process by how the drug was assembled with protein corona, charge on nanoparticle, ionic strength, and the concentration of loaded drug molecule (Kah et al. 2012; Hazra et al. 2010a, b).

9.6.2 In Vivo Protein Corona

In vivo protein corona is an experimental testing condition and is an important strategy toward accurate prediction of nanoparticle's biological fate. Nanoparticles attached with protein should have to target the specific cell for its proper function. There are many reports already been presented about the mistargeting of nanoparticle in the biological systems and also caused cytotoxicity. Sakulkhu et al. (2014a, b) in their study solved this problem by using polyvinyl-alcohol-coated superparamagnetic iron oxide nanowires (SPIONs).

These designed nanowires were delivered to rat models for the investigation of in vivo protein corona formation and their further cellular processing. This study demonstrated that there are notable differences between the in vitro and in vivo protein corona formation and their cellular responses. It has been found in several reports that in spite of having 50% of common proteins on the surface of positive and neutral nanoparticles in the in vivo and in vitro conditions, there were only 8%

similarities for the negative nanoparticles (Sakulkhu et al. 2014a, b; Hazra et al. 2010a, b).

9.6.3 Personalized Protein Corona (PPC)

Personalized medicines are those which are used toward highly target-specific even those for a single patients in particular disease prevention. Use of enzyme-NP coronas for the treatment of specific disease make them categorized by bio-identity. Hajipour et al. (2015) identified the impact of human plasma on the development of diseases and their treatments as the human plasma is a source of proteins which is also used in corona formation. It was demonstrated in their study that the type of protein composition in corona formation get varied upon human diseases which is going to be treated. They introduced the personalized protein corona (PPC) as a new concept in the field of nanobiotechnology. Interestingly the result demonstrated different cellular responses for the exact same type of nanoparticles assembled with various corona structures (Hajipour et al. 2015; Hazra et al. 2011).

Caracciolo et al. were also able to demonstrate the use of differential protein bindings with nanoparticle to form specific corona for therapeutics and identified relationships between the disease state and specific use of nanoparticle (Caracciolo et al. 2014; Hazra et al. 2015). These findings represented a promising aspect of protein corona development for both personalized diagnostics and successful treatments of disease. According to the research works still developed on PPC, scientists could design efficient, safe, and patient-specific enzyme-coated nanoparticles for the treatment of particular disease with successful clinical application (Caracciolo et al. 2015; Hazra et al. 2014).

9.6.4 Gene Therapy

Gene therapy becomes one of the most promising strategies for treating genetic diseases such as mitochondrial-related diseases (Tachibana et al. 2013), blindness (Mancuso et al. 2009), muscular dystrophy (Mendell et al. 2010), cystic fibrosis (Burney and Davies 2012), and certain cancers (Harris 2010; Gustafson and Ghandehari, 2010). However, the lack of ideal delivery systems for the success of gene therapy is now a tremendous challenge specially for the DNA-based gene therapy. However, over the past decade, considerable advancement has been reported in case of polymer based DNA delivery systems for gene therapy. DNA delivery system for gene therapy requires a single DNA carrier. Recently enzyme-NP corona becomes a significant and promising carrier of DNA. Several factors are involved for a suitable DNA transfer inside the body during gene therapy which are appropriate materials engineering i.e., target-specific polymer-DNA construction, surface modification of nanoparticles, etc. Along with good carrier properties, there should also several functional and environmental aspects which are important for a

successful DNA delivery. These are factors are: there should be a reliable protection for the DNA that has to be delivered, excellent colloidal stability should be mentioned in the biological fluids, there should be high cellular uptake capacity and efficient escape ability of the cargo (enzyme-NP-DNA complex) from the degradation by lysosome. Apart from these factors, the cargo should have effective intake into the nucleus and after that DNA unpacking should occur in an organized and errorless way. Above all of these factors, the most important is a successful interaction between the enzyme-nanoparticle corona and DNA.

There are also some hindrances during the DNA delivery in gene therapy among which the most significant is the frequent blockage of DNA bound enzyme-NP corona in reticuloendothelial system (RES). One of the promising strategies for reducing the aggregation of DNA bound enzyme-NP corona in RES and making the clearance is reported to induce by serum component interaction which form hydrophilic corona with polyethelene glycol (PEG), poly(*N*-(2-hydroxypropyl) methacrylamide) (pHPMA), polysaccharides, oligosaccharides or proteins (Elzoghby et al. 2012; Johnson et al. 2011; Li et al. 2014; Tian et al. 2011; Veiman et al. 2015). Besides the mechanisms and challenges of using enzyme-NP corona as DNA delivery vectors mentioned above, increasing attention has been now given and extensive researches are going on worldwide to access enzyme-NP corona as an effective delivery vector which could carry DNA for successful gene therapy. Various physical forces could contribute to the affinity between enzyme-nanoparticle corona and DNA such as Van der Waals interactions (VDW), electrostatic interactions, hydrogen bonding, and hydrophobic interactions (Mahmoudi et al. 2016). There are several reports documented where it is mentioned that if DNA carriers are not designed correctly, then the formation of enzyme-NP corona could elicit negative impacts such as irregular and uncontrolled biodistribution of the DNA carrier result from mistargetting. This further causes cellular toxicity and low therapeutic efficacy (Mahmoudi et al. 2016). Many factors such as surface charge of nanoparticles and the receptor enzymes, size of nanoparticles, hydrophilicity/ hydrophobicity of both the nanoparticles and enzymes, functionalization and coating of the carriers' could affect the DNA delivery by enzyme-NP corona (Walkey et al. 2012). For example it was evident from a study that DNA adsorption capability of the receptor enzyme can increase with the increment of nanoparticle's surface charge. Positively charged nanoparticle surface induce the adsorption of proteins like albumin with isoelectric points ($pI < 5.5$), while negative charged nanoparticle surface prefers absorbing proteins like IgG with $pI > 5.5$ (Aggarwal et al. 2009). The adsorbed proteins also are shown to become denatured by varying the surface charge of the nanoparticle but the neutral-charged nanoparticle surface was reported to retain the adsorbed protein in its intact structure (Lynch and Dawson 2008). A hydrophobic surface of a nanoparticle was reported to absorb more protein than that of a hydrophilic one (Cedervall et al. 2007a, b), therefore it is suggested that avoiding the hydrophobic surface of nanoparticle is a good choice in the construction of carrier for DNA during gene therapy. Because of this the chance of nonspecific accumulation of enzymes on nanoparticle surface reduces and hence possibility of mistargetting of DNA carrier also comes down.

9.6.5 Enzyme Immobilization

Enzyme immobilization is among the new type of nano-polymer based technologies in the recent past years which is aimed to obtain maximum biocatalytic reaction of the immobilized enzyme in industrial, biomedical, and biotechnology sectors.

Nanoparticles based immobilization have three important features: (1) nano-enzyme particles are easy and safe to synthesize as there is no need of surfactants and toxic reagents, (2) homogeneous polymer can be performed, and (3) particle size of nanomaterials can be maintained.

Magnetic nanoparticles are used in enzyme immobilization and effective outcome of the designed system were reported in biotechnological applications (Bayramoglu et al. 2008). It was found that the high surface-to-volume ratio of the magnetic nanoparticles helps in high binding capacity and increased activity of the conjugated enzymes (Konwarh et al. 2009). Cholesterol oxidase is an enzyme which was conjugated with Fe_3O_4 nanoparticles and was applied in the determination of total cholesterol in serum (Kouassi et al. 2005). Kalkan et al. (2009) in their study constructed the enzyme-NP complex between laccase and chitosan-magnetic nanoparticle and successfully demonstrated its use in the bioremediation of environmental pollutants. Konwarh et al. (2009) synthesized keratinase conjugated with iron oxide nanoparticle complex which was used in the synthesis of keratin. The enzyme α-amylase was combined with cellulose coated magnetic nanoparticles for the effective degradation of starch (Namdeo and Bajpai 2009). Ansari and Husain (2011) used ZnO nanoparticles to construct a complex with the enzyme β-Galactosidase for lactose hydrolysis. The enzyme lipase was embedded in the surface of iron oxide nanoparticle for the potential hydrolysis of pNPP, which causes the liberation of inorganic phosphate (Huang et al. 2003).

9.6.6 Enzyme-NP Corona as Biosensor

Nanoparticle are also now been used as biosensor of several types. This biosensor works as a nano-biodevice where enzymes and NPs are embedded on microchips that contain a network of nanowires. Biological catalysis and all the chemical reaction takes place after loading of samples according to the enzymes used and test wanted to perform. The output of the enzyme-mediated reaction is displayed on a digital screen. Many types of enzymes which regulate specific biochemical pathway are used in developing biosensor along with nanomaterials. Biosensors are extensively used in disease diagnosis and different types of biochemical testing both in pathology and industrial sectors.

Zhang et al. (2004a, b) in their study reported the use of enzyme glucose oxidase bound with selenium NP as biosensor for the detection of H_2O_2 and free glucose present in body fluids, food, and agricultural products. The same research groups also demonstrated the use of enzyme uricase complexed with ZnO NP as a biosensor of uric acid (Zhang et al. 2004a, b). Wu et al. (2009a, b) and Jang et al. (2012) have also exhibited glucose oxidase as a glucose biosensor. In their study platinum,

chitosan, and silica NPs were used for embedment of the enzyme. Zhao et al. (2007) and Radhakumary and Sreenivasan (2011) carried out a study where they claimed glucose oxidase as a biosensor for detection of glucose in urine by naked eye. For the development of this biosensor they used platinum and gold NPs. Crespilho et al. (2009) and Sahoo et al. (2011) showed the activity of enzyme urease embedded on silver NP and phosphonate grafted iron oxide NP as a biosensor for urea content determination in blood, urine, alcoholic beverages, natural water, and environmental wastewaters. Lin et al. (2007) presented the enzyme Peroxidase composite with gold-chitosan NP as a biosensor for measuring rapid deterioration of H_2O_2 which is ultimately used in water treatment, pharmaceutical, and biomedical applications.

9.7 Challenges of Using Enzyme-NP Corona

In spite of having several beneficial aspects, there are a numbers of challenges associated with the use of nanoparticles. Still there is needed a prolonged and in-depth understanding on the kinetics and biological effects of nanoparticle binding with the proteins (and other molecules) that construct nanoparticle-based corona. Researchers should have provided with a guideline to perform basic experiments to investigate the development of nanoparticle-protein corona complexes and their biological effects. The interpretations of the beneficial effects of nanoparticles in protecting biological systems are often unclear due to the conflict in various reports. Nonspecific targeting by enzyme-NP corona causes severe systemic toxicity in blood and normal organs. Thus more investigations should be performed for the development of specific cellular targeting strategies to overcome the barrier of toxicity prior to clinical trials. Blood–brain barrier (BBB) has been reported as another exhausting challenge during the delivery of DNA to the brain with the help of enzyme-NP corona.

BBB is characterized by extremely tight junction which is present within brain capillaries endothelial cells (BCECs) and that cells selectively prevents the transport of such nanoparticles through the tight junction (Obermeier et al. 2013; Wohlfart et al. 2012). Therefore, BBB block therapeutics nanomedicine to reach the central nervous system toward the prevention of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and even brain tumors (Chen and Liu 2012). The oral route of drug delivery is preferred mostly so far and it has been found that 70% of drugs are administered orally. Although, many authors have studied the potential use of enzyme-NP corona in oral drug delivery, there have some complexities of the NPs transportation in the gastrointestinal (GI) tract. Orally delivered enzyme-NP corona loaded with drugs has to face several hindrances such as: (1) harsh GI environment, (2) mucosal barrier, and (3) the intestinal enterocyte lining. Researchers have studied and found that due to such complexities, physicochemical features of some NPs lose completely. In addition, some enzyme-NP corona has shown to nonspecifically bind with mucosal and enterocyte lining in gut which cause rapid change in the colloidal stability of those enzyme-NP corona. All these complexities finally result into the decrease in druggability of the lead

molecules that would be carried by enzyme-NP corona to the target site (Berardi and Baldelli 2019).

9.8 Recent Development in Gene Delivery Systems

Recently, some progressive development has been attained in enzyme-NP corona-mediated gene delivery. There are some chemicals that have been used in combination with enzyme-NP corona in purpose of gene delivery. Polyethelene glycol (PEG) is one of them which have shown protective effect in minimizing nonspecific interactions between the enzyme-NP corona with other cellular receptor and the lysosomal degradation of corona during the gene delivery system. PEG acts as a hydrophobic sheath over the corona containing the DNA to be delivered at the target cell during the gene therapy.Importantly, it was noticed in MTT assays that the PEG conjugation with the enzyme-NP corona during the DNA delivery significantly reduce the cytotoxicity and also improve the gene expression efficiency of the delivered DNA in the receptor cell (Zhang et al. 2010). Kleemann et al. (2005) in their work achieved effective DNA delivery to the lung via coupling of some peptide molecule with PEG.

9.9 Conclusion

Enzyme-NP corona in nanotechnology is now being used in many different medicinal aspects. On the other hand, the uses of nanomaterials in biomedical fields have been quite limited in some aspects and there are only few FDA-approved products in market like Abraxane, which is a protein, conjugated with paclitaxel which is used as chemotherapy in cancer treatment. The principal reasons for this type of restriction in nanomedicine are the complications associated with the protein corona. Such complications in this perspective not only increase the conflicts in the nanotoxicology knowledge but also create gap between in vitro and in vivo results. Therefore, a comprehensive understanding and suitable design of nanomaterials with varied functionalities is required. Such understanding and in-depth knowledge would help in designing more potent and selective enzyme-NP corona which can be used as nano-drug delivery and therapeutics against life-threatening diseases. Other than biomedicine enzyme-NP corona could also be used in gene delivery to cure genetic disorder, biomarker in immunotoxicity and cytotoxicity assay, enzyme immobilization and also in disease diagnosis in medicinal and industrial fields. More research in this fields and utilization of such highly efficient enzyme-based nanomaterials can further assist to develop more potent drug delivery systems and also open up different applications in various other sectors.

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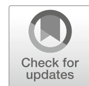

Enzymes in Fuel Biotechnology

10

Bahaa T. Shawky, Neveen B. Talaat, and Sonali Mohapatra

Abstract

Rapid depletion and drawbacks of conventional energy sources worldwide presents a dire situation demanding a potential replacement to surmount the current energy crisis. Lignocellulose which presents a logical candidate for biofuel production has however a high processing cost for alternate bio-based fuels worldwide. For economically feasible bioethanol production, three major aspects which are: feedstock pretreatment technology, enzymatic hydrolysis followed by the configuration of fermentation is to be considered. The pretreatment is a crucial step during lignocellulosic biomass processing, since it not only has a great impact on final yields, but also makes an important contribution to overall costs. Microbial lignocellulolytic enzyme systems are of importance both to the scientific as well as commercial world due to incipient applications in bio-based fuels and other valuable commodity chemicals. Lignocellulolytic enzyme complexes play crucial role in hydrolysis of lignocellulosic biomass and wide range of lignocellulolytic enzymes are thereby of utmost importance in the transition of the society into a more sustainable and bio-based economy. Further, the fermentation processes of lignocellulosic

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hydrolysate such as simultaneous saccharification and fermentation (SSF), separate hydrolysis and fermentation (SHF), consolidated bioprocessing (CBP), and simultaneous saccharification and co-fermentation (SSCF) have to be selected carefully for valuable solutions to the increasing liquid fuel demand. These strategies in future are anticipated to play a major role for replacing oil-based refineries with lignocellulosic-based biorefineries in future.

Keywords

Lignocellulosic biomass · Biological pretreatment · MFEX pretreatment · Cellulases · Hemicellulases · Xylanases · Bioethanol

Abbreviations

AFM	Anaerobic fermentation method
β-Gs	β-glucosidase
β-Xs	β-xylosidase
C1	Swelling factor
CBD	Cellulose binding domains
CBH	Cellobiohydrolase
CBP	Consolidated bioprocessing
CMC	Carboxymethyl cellulase
Cx	Hydrolytic enzymes
DMC	Direct microbial conversion
DP	Degree of polymerization
EG1	Endoglucanase
Exo-G	Exoglucanase
GHG	Greenhouse gas emissions
Li P	Lignin peroxidase
MFEX	Multipurpose fiber expansion
Mn P	Manganese peroxidase
SCM/SeqF	Sequential cultivation method
SHF	Separate hydrolysis and fermentation
SLH	S-layer homology
SmF	Submerged fermentation
SSB	Solid substrate bioconversion
SSCF	Simultaneous saccharification and co-fermentation
SSF	Simultaneous saccharification and fermentation
V P	Versatile peroxidase

10.1 Introduction

The onset of energy crisis along with the environmental globally, has steered to the employment of novel strategies for finding alternative renewable biofuels such as bioethanol, biobutanol, biodiesel, and bio-hydrogen. In this context, the emerging alternative and renewable energy sources as lignocellulosic biomass are expected to play an important role for production of these biofuels. These renewable biomass apart from being green energy resources are also abundantly available that can be utilized for production of biofuels that impart lower environmental pollution and simultaneously meet the future energy demands. Further, the lignocellulosic biomass is relatively inexpensive and do not interfere with food and feed cycles of the human community. The lignocellulosic biomass includes agricultural residues, forest products, energy crops, agro industrial wastes, and municipal solid wastes, etc. and about 2×10^{11} metric tons of carbon and 3×10^{13} J of energy (Ten times the total energy presently consumed in the world) are fixed annually by these photosynthetic green plants (Sánchez and Cardona 2008; Lynd et al. 2008).

Lignocellulosic biomasses are heterogeneous materials that are recalcitrant in nature. The cell wall of these biomasses consists of polymers: cellulose, hemicellulose, and lignin that are interconnected through a complex matrix. Thus, the transformation of lignocellulose into biofuels involves a pretreatment step followed, by enzymatic hydrolysis, fermentation, and eventually the downstream processing strategies. Pretreatment is a crucial step for removal of the recalcitrant lignin from the cell wall of the plant biomass and is observed to be the crucial challenge for large-scale production of biofuels from cellulosic biomass. Followed by the pretreatment, enzymatic hydrolysis that involves the conversion of cellulose and hemicellulose into monomeric sugars by utilization of enzymes also serves as an important factor for the production of biofuels.

Due to the complexity of lignocellulosic materials diverse enzymes are prerequisite for the optimal hydrolysis of lignocellulosic materials. Cellulases (endoglucanases, exoglucanases, and β-glucosides), xylanases and ligninases are the enzymes that are vital for the conversion of lignocellulosic biomass into valuable products. The synergistic action of the enzymes facilitates the breakdown of the lignin component of the cell wall by ligninases with subsequent hydrolysis of cellulose and hemicellulose to monomeric sugars (Himmel et al. 2007). Furthermore, the utilization of lignocellulosic materials for biofuels also demands high enzyme loadings that aids significantly to the noneconomical production of these biofuels (Johnson 2016). Thus, research has now been focused for enhancing the production competence of cellulolytic enzymes. The targeted areas have been mostly selection of robust microorganisms that are capable of secreting high and diversified amount of enzymes or designing of genetically modified enzymes that can be utilized for higher enzyme concentrations (Thomas et al. 2016). Consequently, efforts to harvest local on-site cellulolytic enzymatic cocktails using waste biomass has also been reported (Liu et al. 2013), which appears a feasible alternative for economical lignocellulosic fuel.

The enzymatic saccharification process produces monomeric sugars that aid as substrate for numerous fermentative microbial biosynthetic routes for production of biofuel. The fermentation of the cellulosic biomass by microbes can be conducted by one of the many routes such as simultaneous saccharification and fermentation (SSF), separate hydrolysis and fermentation (SHF), consolidated bioprocessing (CBP), and simultaneous saccharification and co-fermentation (SSCF). Optimal fermentation strategy imparts a significant role in the efficient production of the end product metabolites. Thus, it is important to consider the routes of the fermentation process depending on the substrate used and the end product metabolite required from the process.

With these insights the present review discusses about the importance of lignocellulosic biomass for biofuel production and the biorefinery-based processing techniques for biofuel production from biomass (Table 10.1). The chapter also highlights the strategies for in-house production of enzymes involved in lignocellulosic biomass degradation with a brief discussion on the future aspects of biorefinery-based biofuel production from lignocellulosic biomass.

10.2 Lignocellulosic Biomass as a Vital Renewable Resource for Sustainable Production of Biofuels

Lignocellulosic biomass which are derived from plant material are composed of carbon, hydrogen, oxygen, nitrogen, and minor amounts of minerals. The cell wall components i.e., cellulose, hemicellulose, and lignin show unique structural properties for the lignocellulosic biomass. Cellulose (crystalline structure) is a polymer of glucose molecules and is the most abundant component of lignocellulosic biomass. It comprises of 35–45% of the feedstock. Hemicellulose, is the second abundant (25–35%) branched sugar that consist of the sugars such as arabinose, galactose, mannose, and xylose. Hemicellulose is hydrolyzed quickly due to its amorphous and branched nature. The recalcitrant and the outer fraction of the cell wall of lignocellulosic biomass is lignin (10–25%) that is a complex organic aromatic heteropolymer (Chen and Dixon 2007). It provides strength to the lignocellulosic biomass and hinders the action of hydrolytic enzymes by acting as a barrier.

Apart from the composition, its vast availability, low-cost biomass, renewable nature, potential to reduce greenhouse gas emissions (GHG) are some of the attractive features that imparts the importance of lignocellulosic biomass as sustainable source for production of liquid fuels. Thus, renewable resources are an important area in the search for alternatives to fossil-based raw materials. Nevertheless, energy from biomass will share a crucial part of the solution alongside other sustainable resources such as solar, wind, ocean/hydro, geothermal, hydrogen technology, hydraulic power, and nuclear energies. In fact, inexpensive transformation of biomass into liquid fuels and commodity chemicals will be essential if society is to sustainably and economically meet such needs. The primary barrier in the economical processing of cellulosic biofuels is the recalcitrance or resistance to

Table 10.1 Types of biofuels (Biofuels are categorized into four classes on the basis of biomass utilized)

Feedstock	Products	Biofuel types	Advantage	Technical barriers
Sugar/starch-based crops Oil-based crops	Bioethanol Biobutanol Biodiesel Biogas, vegetable oil	First generation (Sims et al. 2010)	• Agriculture will not only play a role in food production, but also in energy provision • Biofuels have socio-economic impacts	Limited use due to: • Food security issue • Battle for land, water and fertilizers • High processing costs
Nonfood crops, agricultural residues, forest biomass, municipal solid wastes, kitchen waste, newspaper, energy crops	Bioethanol, biobutanol, biohydrogen Bio-oil Wood diesel Bio-Fischer–Tropsch diesel	Second generation "Advanced biofuel" (Bansal et al. 2011)	• Vast range of feedstock with reduced costs • Reduced greenhouse gas emissions • Decreased air pollution • Job creation • Decrease dependency from crude oil imports • Provide more energy in comparison to first generation biofuels • Grown on a poorer quality land with limited water and nutrients	• These fuels cannot be used in unmodified engines above small blends and are not applicable to the jet fuel market • The energy crops still compete with production of fiber and food crops
Engineered algae Algal-to-biofuels technology Algae-based biodiesel	Biodiesel	Third generation "Algae biofuel" "Oilgae" (Mamo et al. 2013)	• Low-cost, renewable and high energy • No competition to food and fiber crops • Wide range of valuable products	
Metabolically engineered algae. Algal-to-biofuels technology but methodology is different. Genetically modified microbes that convert CO_2 in	Biogasoline	Fourth generation (Lu et al. 2013)	• Metabolically engineered algae can capture and store large amounts of carbon	• Let's skip ahead to future biofuel technologies that, if they work, really could signal the beginning of the end of oil

(continued)

Table 10.1 (continued)

Feedstock	Products	Biofuel types	Advantage	Technical barriers
the atmosphere directly into usable fuels Created using petroleum-like hydroprocessing or advanced biochemistry or revolutionary processes "Solar-to-fuel" [sunlight + waste CO_2 + engineered microorganisms] "Solar converter"				

degradation offered by the lignocellulosic biomass. Overcoming this recalcitrance is the central challenge to large-scale commercial production of cellulosic biofuels (Lynd 2010).

10.3 Processing of Lignocellulose Biomass

Biological conversion processes of lignocellulosic materials include: feedstock handling, pretreatment, enzymatic hydrolysis, fermentation, product recovery, by-product utilization, and waste treatment. In addition, these processes are designed with other process streams within the biorefinery concept.

10.3.1 Pretreatment of Lignocellulosic Biomass

The concept of "pretreatment" arose from the observation that if lignocellulosic biomass is subjected to suitable potent pretreatment, enzyme cocktails were much more effective at releasing sugars from biomass. The useful effects of such pretreatments are due to alteration of the cellulose, hemicelluloses, and lignin structure, which will be more susceptible to enzymatic hydrolysis than in their native condition. The primary role of pretreatment is to disrupt the matrix of polymeric compounds that are physically and chemically bonded within lignocellulosic biomass cell wall structures. These compounds include cellulose microfibrils, hemicellulose, and lignin. Unfortunately, each pretreatment has some inherent limitations. For example, loss of sugars, high costs, less safety, causes dehydration of sugars to toxic compounds such as furfural and hydroxymethyl furfural that inhibit subsequent hydrolysis and fermentation, and the need for neutralization, which leads to waste

disposal concerns. Hence, the development of a cost-effective pretreatment technology that separates sugars from all other components in feedstocks is therefore the highest priority for providing valuable insights that will foster the development of a deeper understanding of biomass conversion to biofuels and lead to low-cost pretreatments that will facilitate commercialization of biomass conversion processes with important societal and environmental benefits.

The key structural barricades for the biodegradation of cellulose are its linkages with the lignin and hemicellulose, the crystallinity, and its lower surface area. An effective pretreatment should result in high cellulose digestibility, good hemicellulose recovery, minimal capital and energy cost, good delignification, and a high recovery rate of valuable chemicals. Further, majority of pretreatments apart from removal of lignin also facilitate partial disbanding of hemicelluloses, however are ineffective on the recalcitrant cellulose fibrils. Therefore enzymatic hydrolysis is preferred sequentially, for conversion of crystalline cellulose into amorphous state that can be further utilized by microbes for fermentation of value-added products. Numerous methods have been developed so far to enhance the yield of the sugars by pretreatment processes. However, in the present review the discussions have been focused on two pretreatment systems i.e., biological pretreatment and multipurpose fiber expansion (MFEX) pretreatment for bioethanol production.

10.3.1.1 Enzyme Systems in Microorganisms for Biological Pretreatment

The biological pretreatment refers to the use of fungi mainly white-rot fungi, which favors the selective lignin degradation of lignocellulosic biomass and improve its saccharification. Dias et al. (2010) noted that lignin-degrading enzymes such as manganese peroxidase, lignin peroxidase and laccase cleaved lignin molecules and raised cellulolytic and xylanolytic activities. These enzymes therefore display synergy for biological degradation of biomass. Enzymes produced from microorganisms are either membrane bound or secreted free in the medium (Shawky and Hickisch 1984a, b). Microorganisms possess a repertoire of enzymes, which act sequentially to achieve complete hydrolysis of lignocellulosic feedstocks. A key benefit of using enzymes in these processes is their high selectivity.

10.3.1.1.1 Ligninases

The white-rot fungi or Basidiomycetes are commonly found in forest litter or the degraded fallen trees. These microbes are specifically recognized for lignin degradation and are the only group that is proficient for the same. They efficiently depolymerize, degrade, and mineralize the plant cell walls constituents including the recalcitrant lignin. The most widely studied enzymes in this group are manganese peroxidase (MnP), E.C. 1.11.1.13; lignin peroxidase (LiP), E.C. 1.11.1.14; and laccases, E.C. 1.10.3.2, and several other peroxidases (Sharma and Kuhad 2008). LiP is an heme-containing extracellular peroxidase that is dependent on H_2O and has low optimum pH (Bonugli-Santos et al. 2010). LiP oxidizes target biomass by two one-electron oxidation steps with transitional cation radical formation (Dashtban et al. 2010). Owing to their high redox potentials, LiPs have remarkable potential as

an industrial enzyme for lignocellulosic biomass. Similarly, MnP is a heme-containing extracellular peroxidase that has a requirement for Mn^{2+} as its reducing substrate. It is the most common lignin-degrading peroxidases that is produced by almost all wood-decaying Basidiomycetes. The phenoxyl radicals produced can further react with the eventual release of CO_2. The catalytic cycle of MnP is very similar to that of LiP, differing only in that compound II (Kuhad et al. 1997; Sánchez 2009). Another group of ligninases are laccases that are produced by almost all wood Basidiomycetes and few Ascomycetes and are actively involved in lignin degradation (Dashtban et al. 2010). Following laccases are versatile peroxidase (VP) and glyoxal oxidases. VP has been a hybrid between MnP and LiP as it can oxidize Mn^{2+} along with phenolic and nonphenolic aromatic compounds. It remarkably combines the substrate specificity characteristics of the three other fungal peroxidase families. Glyoxal oxidases conversely are observed in bacterial systems that have been proposed to play a vital role in lignin degradation (Kirk and Farrell 1987).

Biological pretreatment of biomass offers advantages such as disruption and not removal of hemicellulose or lignin may be adequate to realize high sugar yields from biomass in enzymatic operations. It has low energy requirement and is eco-friendly process in comparison to non-biological treatment. Nevertheless, biological pretreatment have limited industrial applicability due to relatively slow processing rates with longer pretreatment time. Additionally, the microorganisms involved in the pretreatment may consume cellulose and hemicellulose, hurting product yields. Hence, improvements in the genetic aspects of these microorganisms for enhanced production of the aforementioned enzymes can be a significant area of research for industrially viable biological pretreatment processes.

10.3.1.2 Multipurpose Fiber Expansion (MFEX) Pretreatment

With the limitations observed from biological pretreatment, multipurpose fiber expansion (MFEX) (Bahaa 2017), pretreatment methods which actively focus of industrial and academic research efforts are of much interest. MFEX pretreatment is an unique pretreatment strategy offers the following advantages: does not degrade solubilized carbohydrates; enhancement of monomeric sugar yields during enzymatic hydrolysis >90% recovery from the theoretical; does not inhibit subsequent fermentation step; produces a highly digestible pretreated solid; requires little biomass size reduction; can work in reactors of reasonable size and moderate cost; produces no solid-waste residues; is simple and practical; is effective at high solids loadings; needs low enzyme lodging for hydrolysis step; low-cost decrystallizing agents' recovery systems; and can greatly improve the digestibility (Fig. 10.1).

10.3.1.2.1 Perspectives on MFEX Pretreatment

10.3.1.2.1.1 Efficacy of Biomass Degradation by MFEX

Two protocols for MFEX sample testing efficacy of pretreated biomass on susceptibility to enzymatic saccharification are described. The first protocol describes a small-scale setup employing low solids concentration that easily enables the testing

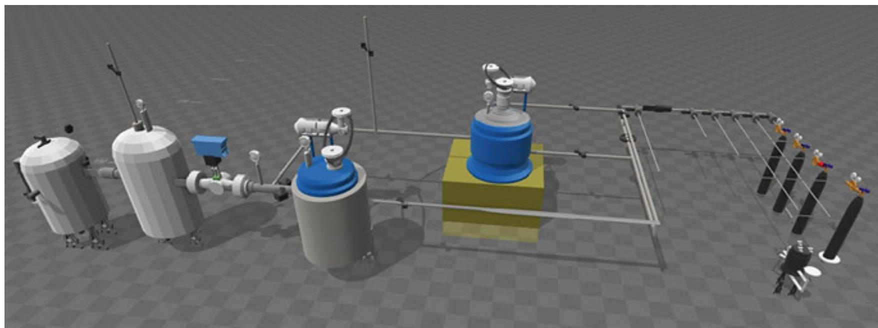

Fig. 10.1 Prototype: Smart MFEX: multipurpose fiber expansion: As an effective, efficient, and eco-friendly pretreatment to improve the production of low-cost fermentable sugars from agricultural biomass and bioethanol as biofuel therefrom. [3D drawing of 200 kg/day vertical pretreatment reactor installed at the National Research Centre Laboratory, Cairo, EGYPT]. (Source: Bahaa 2017)

of a larger number of samples. The second protocol describes a method for testing the efficacy of pretreated biomass at conditions more closely resembling industrial conditions, i.e., high solids concentrations. Both protocols can be used to test the pretreated biomass under a variety of substrate types, substrate concentrations, swelling agent loadings and process conditions, etc. by adjusting the variable parameters of the MFEX process (particle size, moisture content, temperature, stirring, swelling agents used, swelling agents to biomass ratio, pressure, number of flash blow downs, treatment time). On-going techno-economic and lifecycle analysis efforts have shown that integration of the MFEX process into a regional biomass processing can significantly benefit bioeconomies centered on lignocellulosic biomass. Several novel configurations are being developed in parallel with a focus on commercialization to realize cheap, hybrid smart MFEX reactors with in-built low-cost decrystallizing agents' recovery systems.

10.3.1.2.1.2 Biorefinery Based Concepts of MFEX Pretreatment on Lignocellulosic Biomass

The capital cost involved in a pretreatment process is dependent on handling conditions i.e., reaction temperature, residence time, solids loadings, and pretreatment chemical recovery strategies. MFEX pretreatment is an attractive technology from biorefinery concepts that still has to pave its way in the commercial market. The MFEX reactors use decrystallizing agents along with the swelling agents and the biomass as a feed that must be mixed adequately. The MFEX pretreatment is advantageous from a biorefinery concept as swelling agents can be efficiently removed from the treated biomass, recovered, and reused economically. Considering the economic and environmental aspects, restricted pretreatment methods have shown to sustainability fulfill the criteria with their adequate role in commercial scale. In this context, MFEX may serve as an economical pretreatment strategy from a biorefinery point of view due to the possibility of obtaining certain

high value-added products, and have been commercially used to pretreat several lignocellulosic materials (Bahaa 2017). The use of extremophiles microorganisms and/or extremozymes might help in the cost-effectiveness of these processes (Miller and Blum 2010).

10.3.2 Role of Enzymes Involved in Hydrolysis of Lignocellulosic Biomass

Pretreatment of lignocellulosic biomass is followed by enzymatic hydrolysis of lignocellulosic carbohydrate to fermentable sugars and fermentation of the sugars to bioethanol. The hydrolysis of biomass is usually catalyzed by cellulolytic and hemicellulase enzyme system and the fermentation is carried out by specific microorganisms.

10.3.2.1 Cellulases and Hemicellulases

Cellulases are a broad group of enzymes that includes endoglucanases, exoglucanases, and β-glucosides. For efficient hydrolysis of cellulose a high interaction of exoglucanases and endoglucanases is a prerequisite as ideal enzyme mixtures possesses a direct impact over the process economics (Gupta et al. 2009). In this scenario, tailoring of enzymes can be beneficial for environmental and financial safety aspects of the biofuel industry. For industrial enzyme production filamentous fungi and mostly anaerobic bacteria are preferable choices because of their capacity to grow rapidly along with higher titers of enzyme production. Among the fungal species, *Trichoderma* that are well known for their cellulolytic enzyme-producing mechanisms are the most extensively studied species. Similarly, for anaerobic bacteria, *Clostridium thermocellum* that are thermophilic have been best known for their cellulolytic and ethanol-producing mechanisms. These cellulolytic microorganism uniquely produce large extracellular multienzyme complexes called cellulosomes, that can saccharify both the forms of cellulose i.e., crystalline and amorphous forms (Johnson et al. 1982; Shawky et al. 1984). Cellulases have been classified on the basis of their action mechanism into five endoglucanases (EG-1 to EG-5), two exoglucanases or cellobiohydrolases (CBH-1 and CBH-2), and β-glucosidases (β-Gs). Endoglucanase (EC 3.2.1.4) or carboxymethyl cellulase (CMC) mainly attacks amorphous cellulose or soluble forms of cellulose like CMC by randomly cleaving the internal glycosidic bonds, creating new free chain ends. Exoglucanase (EC 3.2.1.91): Exoglucanase (Exo-G) or cellobiohydrolase (CBH) or filter paper cellulase prefers crystalline compounds like avicel. There are two major categories of cellobiohydrolase: CBH I that works processively from the reducing end of the cellulose chain and CBH II that works processively from the non-reducing end of the cellulose chain (Kleman-Leyer et al. 1996). The β-glycosidases (β-G) or cellobiase is inactive against both amorphous and crystalline cellulose and act specifically on the cellobiose disaccharides and produce glucose.

Unlike cellulose, hemicelluloses are a group of heterogeneous carbohydrate polymers including pentoses (xylose and arabinose), hexoses (glucose, galactose,

and mannose), and organic acids (acetic, glucuronic, ferulic, and β-coumaric). Due to the difference in hemicellulose composition, the need for hemicellulolytic enzymes in the cellulolytic enzymes mixture varies with the lignocellulolytic feedstock to be hydrolyzed. It is therefore vital to understand hemicellulolytic enzyme structure to optimally design "enzyme cocktails." Xylan is the most common hemicellulose, that consists of a β-1,4-linked xylopyranose units backbone. The other hemicelluloses are composed of β-1,4-linked mannopyranose along with glucopyranose as backbone. Generally, hemicellulases are placed in three general categories: endo-acting enzymes that attack polysaccharide chains internally; exo-acting enzymes that act processively from either the reducing or non-reducing terminal; and accessory enzymes that help to break down hemicellulose branch chains (Shallom and Shoham 2003). Hemicellulases [endo-1,4-β-D-xylanases (EC 3.2.1.8), endo-1,4-β-D-mannanases (EC 3.2.1.78), α-L-arabinofuranosidases (EC 3.2.1.55), etc.] are responsible for breaking down hemicellulose polymers. Since the hemicellulases are mainly xylan-degrading enzymes the complete degradation of xylan necessitates the combined action of endo-xylanases and exo-xylanases. This synergistic action releases xylobiose, xylooligosaccharides, and β-xylosidases, that further cleaves xylobiose and xylooligosaccharides for the release of xylose molecules.

10.3.2.2 Processes in Enzymatic Hydrolysis

Enzymatic hydrolysis is affected by various factors that subsequently affect the yield and the rate of reaction (Fig. 10.2). The factors such as substrate concentration, enzyme activity, and reaction conditions mostly affect the yield and rate of reaction. Further, the hydrolysis of cellulosic substrates is also dependent on the structural features of the substrate, such as cellulose crystallinity, degree of cellulose polymerization, surface area, and lignin content. The sequence of the major steps of the enzymatic saccharification can be summed up as: (a) binding of cellulase to the substrate via cellulose binding domains (CBD); (b) recognition of the susceptible bonds by cellulases for hydrolysis; (c) formation of the enzyme–substrate complex; (d) movement of the enzyme on the cellulose chain and hydrolysis of β-(1, 4) glycosidic bond; and finally (e) cellobiose hydrolyzed to glucose by β-glucosidase.

10.3.3 Properties of Enzyme Involved in Hydrolysis Process

10.3.3.1 Enzyme Inhibition

Inhibitors such as furans and phenolics produced during chemical and other pretreatment processes are the major inhibitors that affect the enzymatic hydrolysis process. Detoxification methods such as over liming, ion-exchange adsorption, activated carbon adsorption, solvent extraction, steam stripping, and enzymatic (laccase) treatments are effective methods that can be applied for overcoming the enzyme inhibition reactions(Mosier et al. 2005). Further, end product accumulation in active sites of enzymes is also a major reason for inhibition of enzymatic

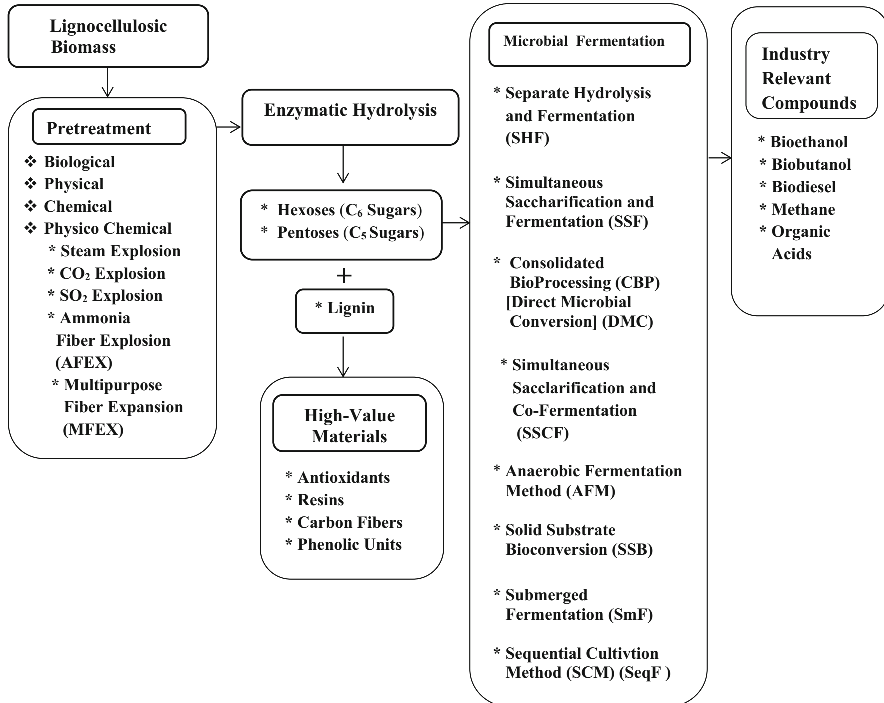

Fig. 10.2 Possible pathways in a lignocellulosic biomass biorefinery platform. (Source: Bahaa T. Shawky NRC—EGYPT/Original)

reactions. For example, cellobiose is a strong inhibitor for cellulose saccharification by cellulases.

Addition of cellobiase (β-glucosidase) is effective in eliminating the cellobiose inhibition. Other end products, such as ethanol, glucose, also inhibit enzymatic hydrolysis but to a lesser extent. However, xylooligomers and xylose have recently been shown to be very strong inhibitors of cellulase.

10.3.3.2 Measurement of Enzyme Activity

Activity is defined as the rate of product formation at a certain temperature, pH, and substrate concentration. Assay values are usually reported as units of activity per mL of aqueous enzyme preparation. The most commonly used assay for comparing cellulolytic enzyme systems is the filter paper assay which measures the hydrolysis of a defined piece of filter paper (Nordmark et al. 2007). However, this assay does not necessarily show the true hydrolyzing capacity of a cellulolytic enzyme system since the activity also depends on the substrate. The standard unit of cellulase activity is defined as the filter paper unit or FPU, which in turn is based on the international unit (IU) defined as 1 mM substrate converted in 1 min. Quantitative cellulase activity assays can be divided into three types: (1) the formation of products

after saccharification, (2) the decrease in substrate quantity, and (3) the change in the physical properties of the substrate. The two basic approaches to measuring cellulase activity are first, measuring endoglucanases, exoglucanases, and β-glucosidases activities, and second measuring the total cellulases activity.

10.3.3.3 Specificity of Enzymes

Specificity is the inherent property of enzymes, and is one of the crucial factors that make enzymes advantageous over the chemical catalysis. It is based on the configuration compatibility between the enzyme and the substrate. The activity of the enzyme is generally represented as International Unit (IU). The specific activity is the number of enzyme units per mg of enzyme Protein. It can be denoted as ($U\ mg^{-1}$). The enzyme activity depends on several factors such as temperature, pH, pressure, etc.

10.3.4 On-Site Production of Cellulolytic Enzymes by the Sequential Cultivation Method (SCM) (SeqM)

One of the major factors that attribute to the high costs of the industrial lignocellulosic biofuel production is high cost of the commercial enzyme cocktails. An alternative strategy for economical production of lignocellulosic biofuel could be on-site production of enzymes, integrated to the biorefinery plant. Thus, laboratory prepared enzymatic cocktails that are prepared using the same lignocellulosic substrate as feedstock for fungal development and biofuels production can be a possible solution (Lynd et al. 2008). Production of enzymes through microbial cultivation can be conducted either by solid substrate bioconversion (SSB) or through submerged fermentation (SmF). Although most of the advances related to the microbial production of cellulases have been developed for SmF, the growth of filamentous fungi, the main producers of cellulolytic enzymes, occurs naturally under conditions similar to SSB. Both SSB and SmF have advantages as well as limitations, that is considered according to the microorganism of interest and the desired product. The SSB offers the advantage of using agroindustrial residues as a cheap carbon source and inducer for microbial enzyme production. A combination of the SSB and SmF cultivation technique, defined as sequential fermentation (SeqF), has been effectively applied for the production of cellulolytic enzymes. The SeqF is conducted by a preculture preparation wherein the fungal growth under SSB condition is conducted as an initial stage followed by a transition to SmF. Furthermore, it has been suggested that use of the enzymes secreted from microorganisms grown on the same lignocellulosic material that will be converted to bioethanol could be a possible means of better modulating the enzymatic complex.

10.3.5 Fermentation

The hydrolysis of lignocellulosic biomass yields reducing sugar, which can be subsequently fermented to bioethanol. Essentially, there are three different fermentation technologies that have been developed by which this can be achieved, namely: Separate hydrolysis and fermentation (SHF), Simultaneous saccharification and fermentation (SSF), and consolidated bioprocessing (CBP) or direct microbial conversion (DMC). SSF has been shown to be the most promising approach to biochemically convert cellulose to bioethanol in an effective way (Wright et al. 1988; De La Rosa et al.1994; Reshamwala et al. 1995). SHF is a conventional two-step process where the lignocellulose is hydrolyzed using enzymes to form reducing sugars in the first step and the sugars thus formed are fermented to bioethanol in the second step. The advantage of this process is that each step can be carried out at its optimum conditions. In SSF, the enzymatic saccharification and fermentation is simultaneously conducted. The main advantages of using SSF for ethanol bioconversion are: enhanced rate of cellulose and hemicellulose hydrolysis due to removal of the sugars that inhibit cellulase activity; lower enzyme loading; higher product yield; and reduced inhibition of the fermenting microorganism in case of continuous recovery of the ethanol. Further, a reduced requirement for aseptic conditions, resulting in increasing economics of the process is also obtained in this process. SSF seems to offer a better option for commercial production of bioethanol from lignocellulosic biomass. CBP or DMC process involves three major steps, namely: enzyme production, hydrolysis of the lignocellulosic biomass, and the fermentation of the sugars, all occurring in one step (Hogsett et al. 1992). The relatively lower tolerance of the ethanol is the main disadvantage of this process. *Neurospora crassa* is known to produce ethanol directly from cellulose/hemicellulose, because it produces both cellulase and xylanase and also has the capacity to ferment the sugars to ethanol anaerobically (Deshpande et al. 1986). Some bacteria such as the thermophile *Clostridium thermocellum* produce a complex cellulosome enzyme structure that may be more effective in hydrolyzing cellulose and hemicellulose into monomeric sugars with the same organism also fermenting the sugars released to final products.

Apart from the type of fermentation, the use of appropriate microorganisms that are capable of fermenting hexosans and pentosans present in the lignocellulosic biomass is an important aspect. Most yeast and bacteria are capable of only fermenting monomers and cannot ferment oligomers to bioethanol. Further, in order to make the process more economical, both pentose and hexose sugars must be converted to ethanol; however, even the most promising fermenting microbes do not efficiently ferment pentoses. As a result, maximizing monomeric sugars becomes vital to maximizing yields. A research strategy currently employed is to convert oligomers to monomers by increasing the concentration of activities that hydrolyze oligomers in enzyme cocktails. Although *Candida shehatae*, *Pichia stipites,* and *Pachysolen tannophilus* can ferment pentose sugars (Abbi et al. 1996), their commercial exploitation for ethanol production is limited because of their low ethanol tolerance, slow rates of fermentation, difficulty in controlling the rate of oxygen

supply and sensitivity to inhibitors generated during pretreatment. Moreover, in mixed sugar fermentation, the pentose uptake is inhibited by hexoses, and thus, the pentose fermentation is only possible at very low glucose concentrations. Genetically engineered microorganisms used in ethanol production have shown significant progress. Besides *S. cerevisiae*, bacteria such as *Zymomonas mobilis* and *Escherichia coli* have been also targeted through metabolic engineering for ethanol production from lignocellulosic biomass (Liu and Hu 2010).

10.4 Future Perspectives

The major goals for future cellulase research would be reduction in the cost of cellulase production and improving the performance of cellulases to make them more effective, so that less enzyme is needed. The former task may include such measures as optimizing growth conditions or processes, whereas the latter requires directed efforts in protein engineering and microbial genetics to improve the properties of the enzymes. Major breakthroughs are needed to reduce the cost of producing the cellulases, and to bring about improvements in their activity and physical properties such as thermotolerance. Combined production of biofuels and bio products could tackle market niches as they can be produced by economical efficient and eco-friendly biorefinery based approach. Nevertheless, further improvements are needed still to make biomass ethanol competitive against gasoline as a transportation fuel.

10.5 Conclusion

Development of cost-effective processes for conversion of lignocellulosic biomass and discovery of novel enzymes for enhanced lignocellulose hydrolysis is one of the main scientific and industrial goals. This could be achieved by applying proteomic strategies for identification of proteins secreted by filamentous fungi that are among the most powerful producers of biomass-degrading enzymes. The latest developments within modern biotechnology, the use of recombinant gene technology, introducing protein engineering and directed evolution, have further revolutionized the development of industrial enzymes, which are opening new avenues for utilization of various lignocellulosic wastes as a source of renewable resources and could solve the problem of waste management as well. Further, enzymatic breakdown of plant feedstock is an essential step for its utilization in biorefinery applications, and the products could serve as substrates for the sustainable and environmentally friendly production of biofuels and commodity chemicals. It is noted that hydrolysis yields are sugar yields, while the fermentation yields are mainly focused on bioethanol yields from monomeric sugars. The assessment of sustainable biomass potential and the evaluation of benefits of biofuels are important key factors for increasing rural energy access. Moreover, the investment to help build capacities in the field for feedstock supply and handling can create favorable

conditions to establishing a biofuel industry. In conclusion it may be said that to solve the technology bottlenecks of the lignocellulosic biomass conversion process, novel science and efficient technology are to be applied, so that bioethanol production from lignocellulosic feedstock may be successfully developed and optimized in the near future.

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Role of Enzymes in Deconstruction of Waste Biomass for Sustainable Generation of Value-Added Products

11

Nisha Bhardwaj, Komal Agrawal, Bikash Kumar, and Pradeep Verma

Abstract

The enzymes and its utility have increased tremendously over the past decade, as the focus presently is diverting toward the development of technologies that are cyclic in nature. This idea depends on the fact that both the substrate and the end product should be biodegradable and should fit well with the idea of it being recycled and reused. The enzymes are biological molecules when used commercially can solve many issues e.g., agro-residues waste disposal, replacement of synthetic processes to natural more environment reliable processes. The effective utilization of agro-residues in biorefinery has been gaining attention but its application has been restricted due to higher lignin content and expensive chemical treatment. The biological delignification involving xylanase, cellulose, and ligninolytic enzymes is an effective method, cheap and carbon neutral as well. These enzymes have wide utility and with the advancement of techniques i.e., protein engineering has enabled the synthesis of enzymes that are industrially feasible, higher production yield and can tolerate harsh conditions. This has widened the application to the areas which were previously not known and were either not possible due to the restrictions. This chapter focuses on different enzymes, the method involved in the production, and its application in the bio-based economy.

Keywords

Agro-residues · Biorefinery · Delignification · Waste biomass

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11.1 Introduction

The bio-based economy revolves around the reuse of waste lignocellulosic biomasses (LCB) which are abundantly available on earth and are renewable (Kim et al. 2019). They have proven to be an effective source for the production of various value-added bioproducts. The LCB is available universally and the sources include trees, agro-residues, industrial by-products, grasses, etc. (Anwar et al. 2014; Isikgor and Becer 2015; Watkins et al. 2015). These LCB consist of the lignocelluloses, which comprises three main components i.e., cellulose (40–50%), hemicellulose (25–30%), and lignin (15–20%) (Gray et al. 2006; Singla et al. 2012). These components together form a three-dimensional network complex structure (Sánchez 2009). Cellulose and hemicellulose in the plant cell wall are less accessible to the microorganisms due to their chemical association, tightly bound interaction with lignin, and their ratio of monomers (Ni and Tokuda 2013; Li and Zheng 2017). The deconstruction of the renewable lignocelluloses for the generation of value-added bioproducts can be done by various physical, chemical, and biological methods, however, as the concerns over the environment conservation is gaining impetuous the use of biological methods are more preferred over other methods (Binod et al. 2018; Wagner et al. 2018; Kumar et al. 2020; Kumar and Verma 2020b). As the LCB has gathered attention worldwide among the researcher's several methodologies for LCB utilization have been developed of which enzymatic hydrolysis is an effective method and environmentally friendly (Chaturvedi and Verma 2013).Thus, keeping in prospect the above points the present chapter focuses on the components of LCB, different enzymes involved in efficient delignification of LCB, mechanism of action of various biocatalyst, methodologies to improve its yield, and its application in the bio-based economy.

11.2 The Lignocellulosic Biomass (LCB) Components

Lignocellulose is a reinforced structure comprising of lignin, cellulose, and hemicelluloses, which are bound together with help of different covalent and noncovalent interactions to form a rigid three-dimensional structure. The three components have been shown Fig. 11.1 and described as follows.

11.2.1 Cellulose

Cellulose is a homopolysaccharide consisting of β-D-glucopyranose moieties linked via β-(1, 4) glycosidic bonds. The amount of celluloses in lignocellulosic materials can range from 30 to 50% of the dry biomass weight (Mussatto and Dragone 2016). Celluloses are mainly located in the secondary wall and the degree of polymerization of cellulose chain can be as low as 10,000 glucose residues in wood and as high as 15,000 glucose residues in cotton (Frederick 2012; Guha et al. 2010). Intra and intermolecular hydrogen bonds result in aggregation of cellulose molecules which

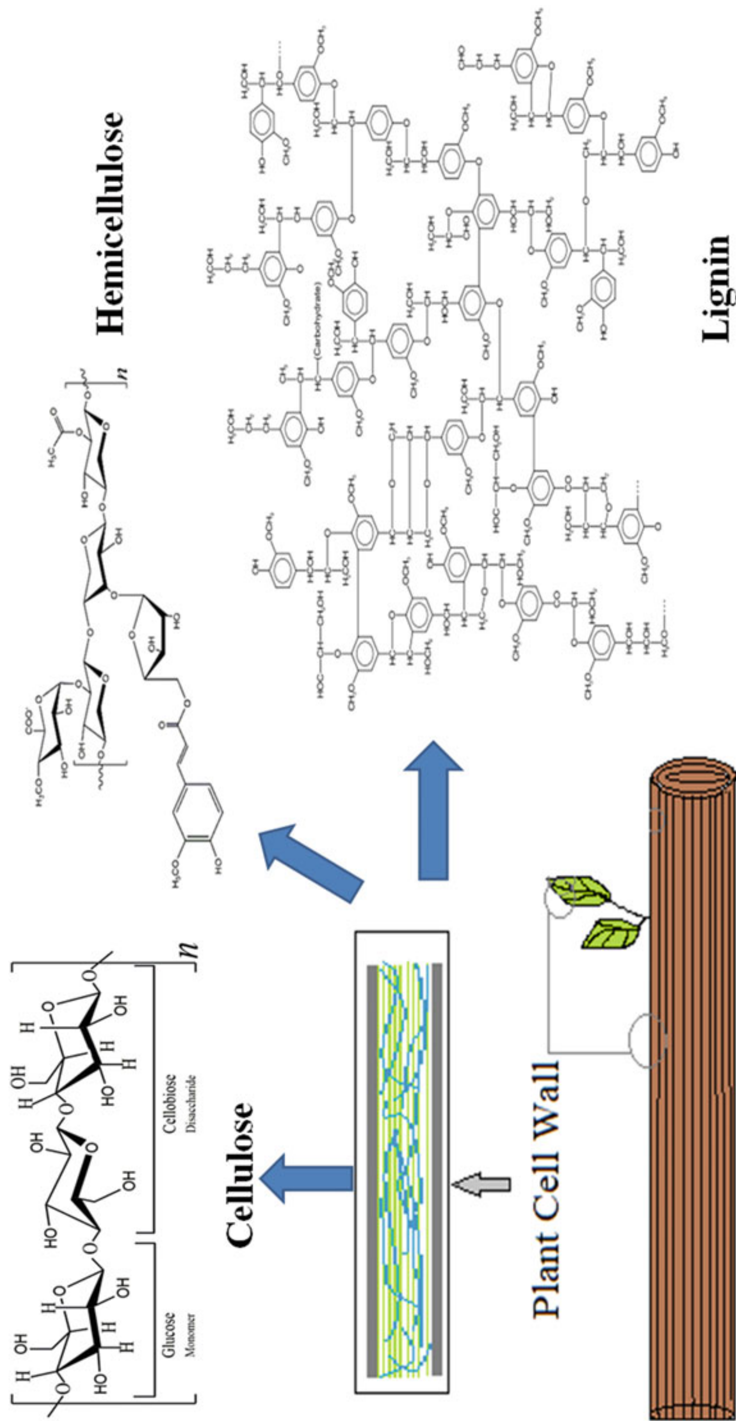

Fig. 11.1 Structural makeup of lignocellulosic biomass (LCB)

are called micro-fibrils that can gather together to form fibrils that subsequently bundle together to form cellulose fibers. The cellulose fibers consist of highly ordered crystalline regions and less ordered amorphous regions as a result of intra- and intermolecular hydrogen bonding. These strong hydrogen bonds and the crystalline structure render cellulose relatively stable toward chemical and enzyme attack and render it insoluble in most of the solvents (Rios-Fránquez et al. 2019).

11.2.2 Hemicellulose

Hemicelluloses are the cluster of various homo and heteropolymers (Perkins 2012). These polymers consist of the main chain of xylopyranose, mannopyranose, glucopyranose, and galactopyranose along with various substituents and together they form a complex polymeric structure (Collins et al. 2005; Shallom and Shoham 2003). Hemicelluloses are mostly found in different plants with varying composition and structure. The major component of hemicelluloses is β-1, 4-xylan, which is the second most abundant polysaccharide in nature after cellulose (Collins et al. 2005; Polizeli et al. 2005; Chávez et al. 2006). Hemicelluloses cover one-third of total renewable LCB found on earth and also comprises around 20–30% of total dry weight of annual plants and tropical hardwood (Collins et al. 2005; Singh et al. 2003).

11.2.3 Lignin

Lignin is made up of phenylpropane units (*p*-coumaryl, coniferyl, guaiacyl, syringyl, and sinapyl) linked with ether and C–C bonds. Phenolic compounds found in lignocellulose are considered as cross-linking agents by ester or ether bonds and by arabinoxylans esterification (Sánchez 2009; Ni and Tokuda 2013). According to Bermek and Eriksson (2009) the 3D structure of lignin is not known, but its composition varies in softwood and hardwood. Softwood comprises of guaiacyl units (14–25% in herbs) and hardwood mainly comprises of syringyl and guaiacyl units (27–32% in woody trees). Higher lignin's with *P*-hydroxyphenyl units along with syringyl and guaiacyl units are found on more woody plants, old trees, and annual plants, deposited in their cell wall which provide rigidity, water proof nature, and protection against microbes.

11.3 The Role of Lignocelluloytic Enzymes in the Bioconversion of LCB to Value-Added Products

The LCB components have the presence of complex structure which requires the activity of various enzymes for its hydrolysis and efficient conversion into monomeric units such as glucose, xylose, *p*-coumaryl alcohol, coniferyl alcohol, sinapyl alcohol, and oligomers such as trioses and tetraoses (Annamalai et al. 2009; Irfan

et al. 2012). Several microorganisms utilizes LCB as their nutrient source and produce several hydrolytic enzymes collectively called as lignocelluloytic enzymes which include cellulase, xylanase, and laccase, lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP). Overviews of these enzymes are described below.

11.3.1 Cellulolytic Enzymes

Cellulases are a multi-enzyme complex which hydrolyzes cellulose. The enzyme complex consists of mainly three enzymes i.e., endoglucanases, cellobiohydrolases, and β-glucosidases. These three enzymes act synergistically on the cellulose polymers to generate glucose monomers (Legodi et al. 2019).

11.3.1.1 Endoglucanases or Endo-1,4-β-D-Glucanase

Endoglucanases (EC 3.2.1.4) hydrolyze internal β-1,4-glucosidic bonds randomly in the amorphous regions of the long cellulose chain resulting in the formation of reducing and nonreducing ends of long-chain oligosaccharides (Singhania et al. 2017; Binod et al. 2018). The crystal structure of endoglucanases (Cel5A) protein of *Thermoascus aurantiacus* consist of a 335 amino acids and these amino acids mold into an eightfold $(\beta/\alpha)_8$ barrel architecture. The catalytic subunit of this enzyme consists of a compact structure containing short loops. Secondary structure of the protein is also represented with a short double-stranded anti-parallel β sheet in β/α-loop$_3$ and 3 one turn helices (Leggio and Larsen 2002). Several biocatalysis of cellulose processes require psychrophilic enzymes and thus study of these enzymes has gained impetus (Violot et al. 2005; Kasana and Gulati 2011). Recently, oxidative type of cellulase has been recognized, this oxidative cellulase utilizes free radical reaction mechanism for depolymerization of the cellulose (Violot et al. 2005; Juturu and Wu 2014).

11.3.1.2 Cellobiohydrolases (CBH) or Exoglucanases

Cellobiohydrolases (EC 3.2.1.91) cleaves the long-chain oligosaccharides releasing cellobiose (Singhania et al. 2017; Binod et al. 2018). The crystal structure of *Clostridium thermocellum* cellobiohydrolase CelS protein folds into an $(\alpha/\alpha)_6$ barrel with tunnel-shaped binding region. At the N-terminal side of the inner α-helices, tunnel cover one-third of the $(\alpha/\alpha)_6$ barrel side and play a crucial role in the substrate binding. Even in the absence of substrate the loops defining the tunnel are stable and thus suggest a dynamic mode of action of cellobiohydrolases (Guimarães et al. 2002).

11.3.1.3 β-Glucosidases or β-Glucoside Glucohydrolases

β-glucosidases (EC 3.2.1.21) hydrolyses the glycosidic bonds of β-D-glucosides or cellobioses and oligosaccharides and generate glucose monomers (Singhania et al. 2017; Binod et al. 2018). Based on the crystal structure of β-glucosidase A (BglA) of *Bacillus polymyxa*, these proteins belong to clan GH-A and GH family. The BglA

protein exists in octameric form, on further screening it was observed that these octamers exist as a tetramer of the dimer in a fourfold axis. These dimmers are strongly stabilized by 14 H-bonds and water molecules. The monomeric units adopt a adopts a single $(\alpha/\beta)_8$ barrel topology and between the α/β subunits secondary structure is inserted (Sanz-Aparicio et al. 1998; Juturu and Wu 2014).

11.3.2 Xylanolytic Enzymes

Xylanases that are also known as "endo-1, 4-β-xylanase" helps in the hydrolysis of 1, 4-β-D-xylosidic bond of xylan (Kuhad et al. 1997; Bhardwaj et al. 2019b). They are the highly preferred catalyst for the endohydrolysis of xylan because of their high specificity, mild reaction condition, and negligible substrate loss. The xylanolytic enzyme can be of six different types, which are as follows.

11.3.2.1 Endo-1,4-β-D-xylanases

Endo-1,4-β-D-xylanases (EC 3.2.1.8) also known as 1, 4-β-D-xylanxylanohydrolase cleaves the glycosidic bonds of backbone of the xylan by reducing the degree of polymerization of the substrates (Kumar and Shukla 2016, 2018). Different xylanases have difference in the substrate specificity which has an important role in the destruction of xylan (Prajapati et al. 2018). The selection of cleaving sites by these enzymes depends on the presence and arrangement of molecules with a specific degree in the side chain and the chain length (Liab et al. 2000; Polizeli et al. 2005).

11.3.2.2 1,4-*β*-Xylosidases

The1,4-*β*-xylosidases (EC 3.2.1.37) plays a very important role after endo-1,4-β-xylanases in xylan degradation by hydrolyzing soluble xylooligomers formed by the endoxylanase hydrolysis of insoluble xylan into xylose from the non-reducing ends (Lasrado and Gudipati 2013). Transglycosylation reactions were also catalyzed by 1,4-*β*-xylosidases in which xylose units release the monosaccharide units or alcohols attached to it (Jordan et al. 2013). Biely et al. (1986) explained the classification of 1,4-*β*-xylosidases, which can be in two forms i.e., xylobiases for xylobiose and exo-1, 4-β-xylanases for larger xylooligosaccharides. Xylobiose is the most suitable substrate for the 1,4-*β*-xylosidases and its xylooligosaccharides affinity depends on the degree of polymerization which is inversely proportional to each other (Terrasan et al. 2016). These enzymes can also cleave some artificial substrates e.g., *o*-nitrophenyl- and *p*-nitrophenyl- c-D-xylopyranoside (Jordan et al. 2013).

11.3.2.3 Acetyl Xylan Esterase

Acetyl xylanesterase (EC 3.1.1.72) primarily hydrolyze the acetyl xylan esters by removing the O-acetyl groups from 2 and/or 3 positions on the β-D-xylopyranosyl residues. Xylan from different plant origin varies in their arrangement of the side chain. The xylopyranoside units with acetic acid were found in the C2 and C3 position in the hardwood and annual plants, 4-*O*-methyglucuronic acid at C2

position in hardwood, softwood, and annual plants and arabinose at the C3 position of softwood and annual plants (Adesioye et al. 2016). The enzymatic approach that cleaves the backbone of xylan can be interfered with due to the presence of acetyl side-groups, acetyl xylan esters play an important role by the steric hindrance and elimination of these groups and facilitating the endoxylanase activity (Polizeli et al. 2005).

11.3.2.4 Arabinase

The L-arabinase is a branched polymer made up of (1-5)-, (1-2)-and (1-3)-α-linked L-arabinofuranosyl residues and its component are occupied with covalent cross-linking of polysaccharides of the cell wall. Mode of action of arabinase differentiate it into two types i.e., *p*-nitrophenyl-α-L-arabinofuranosides and branched arabinans degrading exo-α-L-arabinofuranosyl (EC 3.2.1.55) and linear arabinans hydrolyzing endo-1,5-α-L-arabinase (EC 3.2.1.99) (Semenova et al. 2018).

11.3.2.5 α-Glucuronidase

The α-glucuronidase (EC 3.2.1.139) hydrolyzes the α-1, 2-linkage of glucuronic acid residues, and nonreducing terminal β-D-xylopyranosyl backbone units of glucuronoxylan (aldouronic acids) and releases MeGlcA of GlcA (Yan et al. 2017). Uronic acid and xylose ratio depends on the source of xylan e.g., in softwood the ratio is 1:5 which contains a large amount of 4-*O*-methyglucuronic acids, whereas in hardwood very less uronic acid is found with the ratio of 1:10. Similarly, in annual plants uronic acids quantity depends upon the plants species and it varies even on their parts (Asmadi et al. 2017; Sporck et al. 2017).

11.3.2.6 Ferulic Acid Esterase and *p*-Coumaric Acid Esterase

These enzymes cleave the ester bonds present on the xylan. Ferulic acid esterase (EC 3.1.1.73) between arabinose and side group of ferulic acid and *p*-coumaric acid works with arabinose and *p*-coumaric acid (EC 3.1.1.) (Hunt et al. 2017; Morris et al. 2017).

11.3.3 Ligninolytic Enzymes

The ligninolytic enzymes consist of laccases, hemeperoxidase which are of two types namely lignin peroxidises (LiP) and manganese peroxidase (MnP), and lastly the versatile peroxidises (VP), which are described as follows.

11.3.3.1 Laccase

Laccases (E.C. 1.10.3.2) also known as benzenediol: oxygen oxidoreductase or *p*-diphenoloxidase belongs to the oxidoreductase class. The laccase is universally present however the laccase from white-rot fungi are the most commonly studied. The laccase can break the lignocellulosic wall, complex polyphenol structure which consists of lignin. These enzymes have a broad substrate range, though they do not require an inducible component for its production or enhancement, but considering

the industrial prospect and utility it can be considerably induced in the presence of copper, dyes, or other recalcitrant compounds (Minussi et al. 2007). The enzyme is capable of degrading the phenolic compounds in the absence of mediator, however, the degradation of the non-phenolic compounds require the mediator to initiate the reaction, which is contributed due to the redox potential of both the enzyme and the substrate which plays an important part in the acting capability of the enzyme on various substrates (Kudanga and Le Roes-Hill 2014; Agrawal et al. 2019).

11.3.3.2 Heme-Peroxidases Consisting of MnP and LiP

The heme-peroxidases are an important part of ligninolytic enzymes consisting of lignin peroxidase (LiP) and manganese peroxidase (MnP). LiP and MnP have three reactions of the catalytic cycle which are as follows a) Hydrogen peroxide oxidizes the enzyme to produce compound I (modified enzyme) and water, (b) The compound I (modified enzyme) catalyzes the production of compound II (second modified form of an enzyme), formed by the electron transfer from the reduced substrate along with a generation of free radical, (c) The compound II reacts with the molecule of a reduced substrate to produce another free radical and water. As the reaction continues the enzymes reduces to its native form, (Piontek et al. 1993; Sundaramoorthy et al. 1994; Choinowski et al. 1999; Martínez 2002; Dias et al. 2007; Piontek et al. 1993) and are represented in Fig. 11.2.

11.3.3.2.1 Lignin Peroxidise

LiP's (E.C. 1.11.1.14) were originally discovered in *Phanerochaete chrysosporium* (Dias et al. 2007). LiP possesses high redox potential and can catalyze the degradation of a broad range of aromatic structures. It oxidizes aromatic rings which are moderately activated by electron-donating substitutes whereas the common peroxidases participate in the catalysis of aromatic substrates. An explanation for

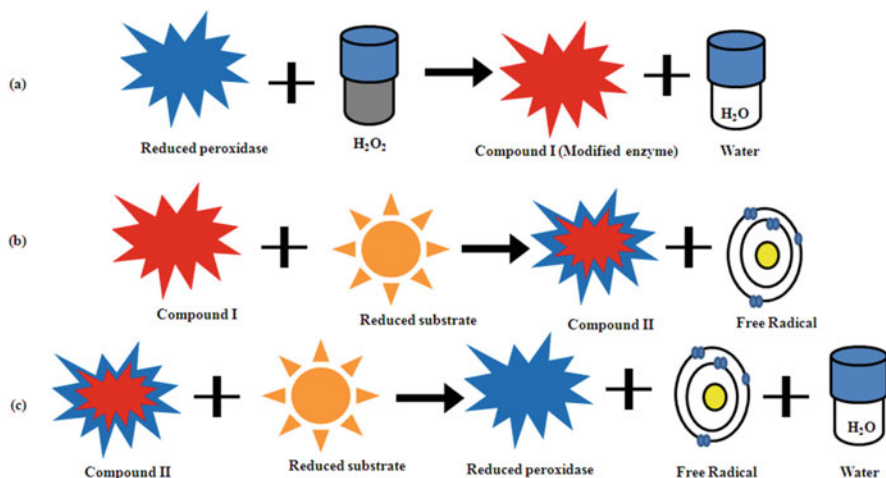

Fig. 11.2 The schematic representation of the three reactions in the catalytic cycle of LiP and MnP

this type of catalysis is the production of veratryl alcohol radicals which have higher redox potential than LiP's compounds I and II and can eventually participate in the degradation of compounds with high redox potential (Khindaria et al. 1996).

11.3.3.2.2 Manganese Peroxidise

MnP (EC 1.11.1.13) are glycoproteins with a molecular weight in the range from 38 and 62.5 kDa (Hofrichter 2002), MnP structure has two domains which are as follows: first heminic group in the middle, second consist ten major helixes, third is a minor helix, and fourth consist of five disulfide bridges. Among those bridges, one bridge participates in the manganese (Mn) binding site, and is this site distinguishes MnP from other peroxidases (Sundaramoorthy et al. 1994). The catalytic reaction has been previously described in Fig. 11.2. However, in case the compound II mandatorily requires the presence of Mn^{2+} for its reaction to proceed. The compound II then oxidizes Mn^{2+} to Mn^{3+} which is responsible to oxidize aromatic compounds. The converted Mn^{3+} are than stabilized by organic acids which react nonspecifically with organic molecules thereby removing an electron and a proton from the substrates (Hofrichter 2002), The attack of the Mn^{3+} which is a small size compound having high redox potential diffuses easily in the lignified cell wall thereby attacking inside the plant cell wall facilitating the penetration as well as the action of other enzymes (Martínez 2002; Hammel and Cullen 2008).

11.3.3.2.3 Versatile Peroxidises

The VP (EC 1.11.1.16) can directly oxidize Mn^{2+}, methoxybenzenes, phenolic aromatic substrates similarly to MnP, LiP, and horseradish peroxidase. VP has a very broad substrate specificity range and can oxidize the substrates even in the absence of manganese. It can efficiently oxidize both phenolic and nonphenolic lignin model dimers (Polak and Jarosz-Wilkolazka 2012). Thus considering the broad range of substrate overproduction system is desired for biotechnological and industrial sectors (Plácido et al. 2013; Hoopes and Dean 2004).

11.4 Mode of Action of Various Lignocellulolytic Enzymes

The mode of action of the lignocellulolytic enzymes has been represented in Fig. 11.3 and described as follows:

11.4.1 Mode of Action of Cellulolytic Enzymes

Cellulolytic enzyme works in synergism and thus cellulose hydrolysis is a stepwise process where the first step is called primary hydrolysis followed by secondary hydrolysis. The primary hydrolysis occurs in the presence of endoglucanases and exoglucanases, these enzymes act on the cellulosic substrate and results in the release of cellobioses. Thus the primary hydrolysis (depolymeriztion) is often regarded as rate-limiting step. The cellobioses are subjected to secondary hydrolysis

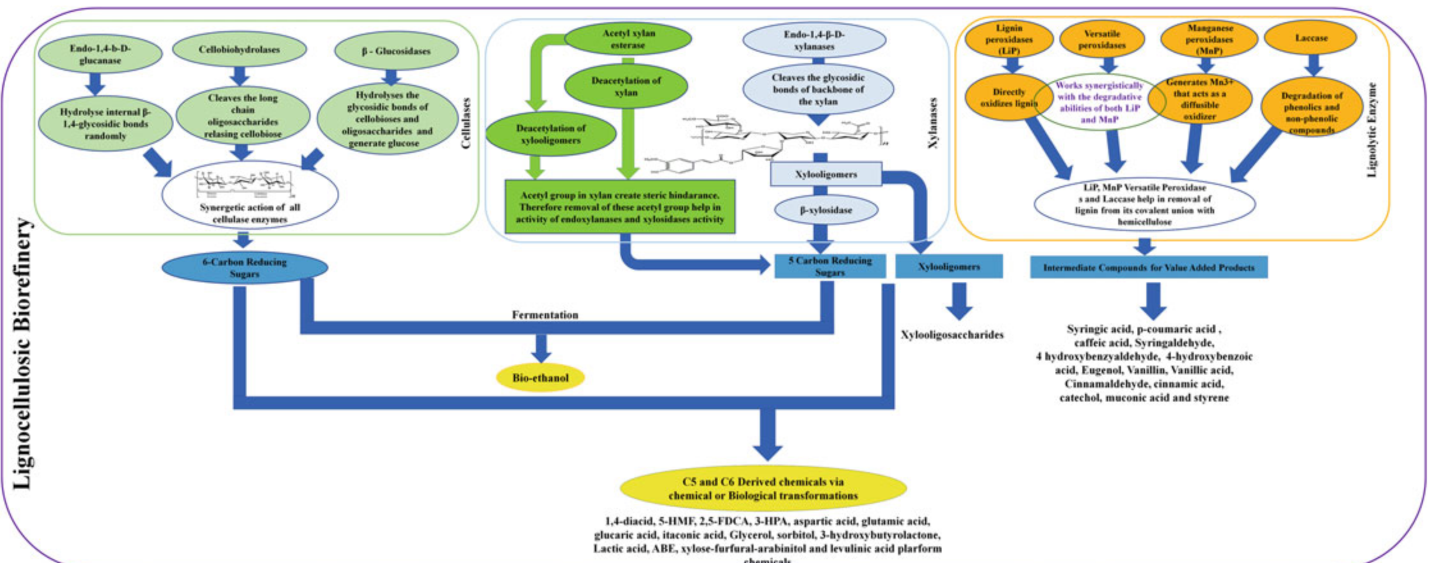

Fig. 11.3 Schematic representation of mode of action and application of lignocelluloytic enzymes in deconstruction of LCB and production of value-added products

in presence of β-glucosidase for the generation of glucose monomers (Kubicek 1992; Binod et al. 2018).

Cellulase enzyme has been broadly divided into two types: non-complex system and complex system. The non-complex system i.e., endoglucanases and cellobiohydrolases have a characteristic two-domain structure a catalytic domain and a cellulose-binding domain (CBD) or carbohydrate-binding module (CBM) (Kuhad et al. 2016). The catalytic domain consists of catalytic site and carbohydrate/cellulose-binding domain as the name suggests help in the binding of enzyme to cellulose and these domains are linked by glycosylated flexible linker peptide. The CBD plays a vital role in ensuring the orientation of the substrate with the catalytic domain for perfect binding and subsequent degradation of cellulose. The complex system consists of high-molecular-weight complexes known as "cellulosomes" (Behera et al. 2017). These complex cellulases are characteristics of the anaerobic bacteria but observed in certain anaerobic fungi as well. The cellulosomes are protuberance from the bacterial cell wall, which harbor stable enzyme complex that causes degradation of cellulose by binding to it (Doi and Kosugi 2004).

11.4.2 Mode of Action of Xylanolytic Enzymes

Xylan hydrolysis done by xylanolytic enzymes either by inversion or retention of the anomeric centers of monomeric units suggesting the participation of one or two chemical transition state (Moreira et al. 2016). During retention two glutamate residues being employed in the catalytic mechanism, which is double displacement mechanism having an acid catalyst protonating the substrate, a carboxyl group situated with a covalent glycosyl enzyme intermediate with the carboxylate in which substrate is opposite to the sugar anomeric configuration. This can reach both sides through transition state including oxo-carbonium ions (Collins et al. 2005; Uday et al. 2016), whereas in inversion mechanism aspartate along with glutamate is involved (Bhardwaj et al. 2019b). This is a single displacement mechanism, in which only one carboxylate in offers for overall acid-catalyzed group departure. This enzyme also acts as a base for activating a nucleophilic water molecule to attack the anomeric carbon (depending upon the distance between two molecules) for breaking the glycosidic bonds and causing an inversion of anomeric carbon configuration (Subramaniyan and Prema 2002).

11.4.3 Mode of Action of Ligninolytic Enzymes

The action of the enzyme on lignin is an oxidative process where the dissimilar fungal oxidases e.g., glyoxal oxidase, pyranose-2 oxidase, and aryl-alcohol oxidase release extracellular H_2O_2, which oxidizes the polymer in a reaction which has been catalyzed by LiP, MnP, and laccase (Martínez et al. 2005; Kersten and Cullen 2007; Hammel and Cullen 2008). The high redox potential of LiP and VP has enabled it to

Table 11.1 Various strain reported for the production of cellulase

Bacteria	Fungi
Acetivibrio cellulolyticus	*Aspergillus niger*
Bacteroides cellulosolvens	*Fusarium oxysporum*
Clostridium	*Penicillium funiculosum*
Ruminococcus	*Penicillium pinophilum*
Pseudomonas fluorescens	*Sclerotium rolfsii*
Bacillus sp.	*Schizophyllum commune*
Cellulomonas	*Trichoderma ressei*
Cellvibrio	*Caecomyces*

act on a wide range of substrates including lignin which oxidizes the benzenic ring irrespective of the degree of methylation and interunit linkages (Ruiz-Dueñas and Martinez 2009), resulting in the formation of unstable aromatic cation radicals, which leads to depolymerization as well as other reactions (Martínez et al. 2005). The released H_2O_2 activates the heme group and enables it to gain a cofactor via an access channel, thus blocking the ability to oxidize lignin as it is unable to reach the site (heme pocket) where oxidation takes place. Thus these enzymes then oxidize the lignin with the help of the bare tryptophanyl radical (Ruiz-Dueñas and Martinez 2009). As the redox potential of laccase is ($\leq$0.8 V), it can directly attack the phenolic moiety which constitutes 10% of the polymer, but the action of a non-phenolic moiety (redox potential is $>$1.3 V in comparison to laccase) the direct action would be difficult. However, this problem can be overcome by the use of redox mediators effectively known as laccase mediator system (LMS) (Ralph et al. 2008).

11.5 Microbial Sources for the Production of Lignocellulolytic Enzymes

Naturally, all living beings are capable of degrading carbohydrates; however, capacity to produce cellulase is limited to microorganisms along with some molluscs. Both anaerobic and aerobic bacteria and fungi are capable of producing cellulase. The anaerobic bacteria are mostly found in soil, in cattle rumens, in the termite gut, decomposing plant materials, and isolated from different paper and wood processing industries wastes (Table 11.1) (Rees et al. 2003). As the application of cellulases in various industrial processes requires stability over ambient temperature it has also led to the production of cellulases from thermophilic fungi e.g., *Chaetomium thermophilum, Humicola grisea, Myceliopthora thermophila, Sporotrichum thermophile,* and *Thermoascus aurantiacus* (Singhania et al. 2017; Binod et al. 2018).

Over the decades many microorganisms such as fungi and bacteria have been reported by many researchers that utilizes hemicelluloses (Table 11.2) (Pokhrel and Yoo 2009; Dong et al. 2012; Liao et al. 2014). Fungi are known as a potential source of xylanases as they produce high titer of extracellular xylanase from various cheap

Table 11.2 Various strain reported for the production of xylanase

Bacteria	Fungi
Bacillus altitudinis	*Aspergillus tamari*
Bacillus mojavensis	*Aspergillus niger*
Bacillus pumilus	*Penicillium purpurogenum*
Bacillus tequilenis	*Aspergillus flavus*
Bacillus licheniformis	*Trichoderma reesei*
Bacillus aerophilus	*Aspergillus welwitschiae*
Bacillus polymyxa	*Aspergillus nidulans*
Pichia stipitis	*Trichoderma longibrachiatum*

Table 11.3 Various strain reported for the production of laccase, manganese peroxidase, lignin peroxidase and versatile peroxidase

Laccase	
Insects	Fungi
Nephotettix cincticeps	*P ostreatus*
Manduca sexta	*Trametes versicolor*
Reticulitermes flavipes	*Cerrena unicolor*
Tribolium castaneum	*Aspergillus flavus*
Bacteria	Plants
Lysinibacillus sp.	*Rhus vernicifera,*
Streptomyces psammoticus	*Pinus taeda*
Bacillus subtilis	*Populus trichocarpa*
Azospirillum lipoferum	*Liriodendron tulipifera*
Manganese peroxidase	
Bacteria	Fungi
Serratia marcescens	*Pleurotus eryngii*
Bacillus pumilus	*Bjerkandera adusta*
Paenibacillus sp.	*Cerrena maxima*
Lignin peroxidase	
Bacteria	Fungi
Pseudomonas aeruginosa	*Phanerochaete chrysosporium*
Bacillus megaterium	*Lentinula edodes*
Serratia marcescens	*Phellinus pini*
Versatile peroxidase	
Fungi	
Bjerkandera sp	
Phanerochaete chrysosporium	
Pleurotus eryngii	
Pleurotus ostreatus	

sources such as agricultural residues by degrading their cell wall (Su et al. 2011). Various mesophilic fungi, some white-rot fungi (Schimpf and Schulz 2018), and filamentous fungi (Bhardwaj et al. 2017) are most commonly reported for xylanase production as they produce stable enzymes.

The ligninolytic enzymes are universally present and present in different types of organisms as plants, bacteria, insects, and fungi (Table 11.3). The presence of

laccase in plants was studied in Japanese lacquer tree, mango, mung bean, peach, tobacco, Zea mays, etc. (Polak and Jarosz-Wilkolazka 2012), bacteria includes actinomycetes, α-proteobacteria, and γ-proteobacteria (Bugg et al. 2011). The presence of laccase has been detected in insects. In the case of fungi, ligninolytic enzymes occur in *Ascomycetes*, *Basidiomycetes*, and *Deuteromycetes* (Dos Santos et al. 2007).

11.6 Various Approaches Implemented for the Enhanced Production of Lignocellulolytic Enzymes

With advent of biotechnology, several pre-existing harmful chemical mediated processes have been replaced by the enzymatic processes. The applications of enzymes in these industries are limited due to low enzyme yield, temperature, and pH stability and cost of production and suitable downstream processing and reusability. Therefore several researchers are trying to overcome these limitations by adopting various approaches which are as follows:

11.6.1 Fermentation Process Used for the Production of Lignocellulolytic Enzymes

The choice of fermentation technology significantly affects the level of production. Submerged fermentation (SmF) is most accepted for lignocelluloytic enzyme production strategy due to extracellular nature of the enzyme. In SmF method, microbes are cultivated in an aqueous medium rich in essential nutrients for microbial growth. However the cost of the concentration and extraction of enzyme is high. Therefore an alternative i.e., solid state fermentation (SSF) was adapted, it involved the growth of microbes on solid materials (moist), and this method mimicked the natural habitat of the microbes (Hölker et al. 2004). However, the easy handling and monitoring associated with the liquid medium have given an upper hand to the SmF over SSF. Different fermentation method employed for lignocellulolytic enzyme production and their yield have been mentioned in Table 11.4.

11.6.2 Lignocellulose as Substrate Used for Enzyme Production

As the cost associated with the production is very high, thus cheap substrates are essential for the enhanced enzyme production. The lignocellulosic substrates such as different agro and forest residues have been used for cellulase, xylanase, and laccase production and help in lignocelluloytic enzyme synthesis without the supplementation other culture medium component and inducers (Elisashvili and Kachlishvili 2009; Ravindran and Jaiswal 2016; Luo et al. 2019; Novy et al. 2019).

Table 11.4 Reported microbial strains, methodology adapted, substrate used for lignocelluloytic enzymes production and its application in various fields

Enzyme	Strain	Method	Substrate	Yield	Properties and application	References
Cellulase	*Sporothrix carnis*	SmF	CC	285.7 U/mL	Enzyme is thermostable at 80–90 °C and suitable for biofuel industries	Olajuyigbe and Ogunyewo (2016)
Cellulase	*Phialophora* sp. G5	SmF	CMC-Na	Specific activity 10.3 U/mg	Stability of 47% at 90 °C	Zhao et al. (2012)
Cellulase (Endoglucanase)	*Aspergillus terreus*	SSF	RS, WS, CS, ZS,J, W BS	141.29 U/g	Enzymatic degradation of delignified RS	Narra et al. (2014)
Cellulase (Endoglucanase)	*Thermoanaerobacter tengcongensis* MB4	SmF	NA	294 U/mg	Stability at a high temperature and is halo tolerant. Suitable for industrial applications	Liang et al. (2011)
Cellulase (Endoglucanase)	*Aspergillus fumigates*	SmF and SSF	WS	SmF: 6294 IU/mg SSF: 9158 IU/mg	Higher specific activity in SSF as compared to SmF	Saqib et al. (2010)
Cellulase	*Schizophyllum commune* NAIMCC - F –03379	SmF	WB	CMCase 195 ± 3.5 IU/mL, FPase 245 ± 1.12 IU/mL	Acid/ alkali and thermo tolerant cellulase efficiently hydrolyzed RS with sugar yield of 1.162 mg/ mL	Kumar et al. (2018)
Cellulase and xylanase	*Aspergillus fumigatus* SK1	SSF	OPT	Endoglucanase (CMCase) 54.27 U/g, exoglucanase (FPase) 3.36, β-glucosidase 4.54 and xylanase 418.70 U/g	Thermotolerant enzyme and saccharification of untreated oil OPT and NaOH-treated OPT gave sugar yield of 8.55 g/land 5.09 g/L	Ang et al. (2013)
Cellulase and Xylanase	*Myceliophthora thermophila* JCP	SSF	SB, SBM, WB and oat	Endoglucanase 357.51 U/g β-glucosidase, 45.42 U/g, xylanase931.11 U/g, avicelase 3.58 U/g	Saccharification of microwave pre-treated SB gave glucose and xylose yields of 15.6 and 35.13%	de Cassia Pereira et al. (2015)

(continued)

Table 11.4 (continued)

Enzyme	Strain	Method	Substrate	Yield	Properties and application	References
Xylanase	*Fusarium proliferatum*	SmF	OSX	Specific activity of 591 U/mg protein	Conversion of xylan to oligosaccharides such as xylobiose and short-chain XOS	Saha (2002)
Xylanase	*Penicillium canescens*	SSF	SOC	18,895 IU/gds	Stable at freezing temperature for 6 months and at room temperature for 3 months	Antoine et al. (2010)
Xylanase	*Clostridium strain* DBT-IOC-DC21	SmF	RS	Ethanol yields 26.61 mM with Xylan, 43.63 mM with xylose, 40.22 mM with mixture of cellulose and xylan and 19.48 mM with untreated RS	Direct microbial conversion of untreated rice straw to ethanol.	Singh et al. (2018b)
Xylanase	*Streptomyces* sp.	SmF	BWX	10,220 U/mL	Purified enzyme could successfully clarify orange, mousambi, and pineapple juice to 20.87%, 23.64%, and 27.89% clarify juices of orange (20.87%), mousambi (23.64%), and pineapple juice (27.89%)	Rosmine et al. (2017)
Xylanase	Aspergillus niger JL-15	SSF	Orange peel	917.7 U/g	Generation of XOS such as xylotriose from birchwood xylan and wheat bran	Dai et al. (2011)
Xylanase	*C. pseudorhagii* SSA-1542T	SmF	Xylan and D-xylose	Activity of 1.73 U/mL with xylan and 0.98 U/mL D-xylose	60.7% fermentation efficiency in 48 h	Ali et al. (2017)
Cellulase and Xylanase	*Aspergillus niger*	SSF	SMB	FPase activity 0.55 IU/g, endoglucanase 35.1 IU/g, xylanase-47.7 IU/g	Hydrolysis of SB yielded 4.4 g/L of glucose after 36 h	Vitcosque et al. (2012)

Xylanase	*Aspergillus niger*LC1	SmF	RS	1245 ± 2.4 IU/mL	Generation of XOS: xylobiose, xylotriose from different untreated agro-residues by partially purified xylanase	Bhardwaj et al. (2018)
Laccase	*Pleurotus ostreatus*	SSF	Na	13,000 U/L	The strain performs better in SmF than in SSF	Téllez-Téllez et al. (2008)
Laccase	*Trametes versicolor*	SSF	SCS	2600.33 ± 81.89 U/g	The SCS improved laccase production and enabled the production of low cost enzyme	Adekunle et al. (2017)
Laccase	*Cerrena unicolor*	SmF	OP	56.3–78.5 U/mL	Removal of phenolic compounds	Elisashvili et al. (2018)
Laccase	*Lysinibacillus* sp.	SSF	SBM as inducer	2.39-fold increase	The phenolic-rich waste can be potential enhancers for the laccase production	Sharma et al. (2017)
Laccase	*Aspergillus flavus*	SmF	NA	15.96 U/mL	An alternate for the valorization of agro-waste	Ghosh and Ghosh (2017)
Laccase	*Myrothecium verrucaria* ITCC-8447	SmF	Glucose	1675 U/L	Delignification of agro-residues	Agrawal et al. (2019)
Lip and MnP	*Phanerochaete chrysosporium*	SSF	SWS	Lip and MnP activity 2600 and 1375 U/L	Effective use of agro-residues for large scale enzyme production	Fujian et al. (2001)
LiP	*Pleurotus* ostreatus (PLO 9) *Ganoderma lucidium* (GRM 117)	SSF	J. curcas L. seed cake	PLO 9-10602.68 U/mL and GRM 117-9419 U/mL	Dye decolorization	Oliveira et al. (2018)

(continued)

Table 11.4 (continued)

Enzyme	Strain	Method	Substrate	Yield	Properties and application	References
VP, laccase	*P. eryngii, P. ostreatus and P. sajor-caju*	SmF	To the media 2,4-DCP was added	The activity was similar for all the three strains 400–700 mU/mL VP, 10–20 mU/mL	The enzyme activity was detected during the degradation of 2,4-DCP	Rodriguez et al. (2004)

RS rice straw, *WS* wheat straw, *CS* cotton, *ZG* zinjivo grass, *BS* bajra straw; *CC* corn cob, *WB* wheat bran, *OPT* oil palm trunk, *SB* sugarcane bagasse, *SBM* soybean meal, *OSX* oat spelt xylan, *SOC* soy oil cake, *BWX* beech wood xylan, *SCS* steam exploded corn stalk, *OP* olive pomace, *SWS* steam exploded wheat straw, *JW* jowar, *SmF* submerged fermentation, *SSF* solid state fermentation, *XOS* xylooligosaccharides, *LiP* lignin peroxidase, *MnP* manganese peroxidase, *VP* versatile peroxidase

11.6.3 Process Optimization for Enhanced Enzyme Production

The enzyme production by microbes are influenced by various nutrient and physical parameters such as substrates, salts in medium, pH, temperature, and light regulation, agitation/shaking the medium (Zhanga et al. 2019). Considering the high cost of commercially available enzymes and increase in their industrial demands, all the above mentioned factors can be optimized using a well-known conventional method One-Factor-at-a-Time (OFAT). However OFAT is time consuming and it can only give the effect of individual component in the fermentation and does not explain about the interaction among them (Deswal et al. 2011; Kumar et al. 2018). Later Response Surface Methodology (RSM) a statistical tool was designed which helped in analyzing multiple factors at the same time (Abdullah et al. 2018; Neelkant et al. 2019). Thus being economically feasible and time saving in contrast to OFAT, in the present time RSM is much more preferred over OFAT.

11.6.4 Strain Improvement for Enhanced Enzyme Production

Several attempts have been made for the improvement of strains by applying techniques such as (a) mutagenesis and selection, (b) gene cloning, and (c) genome shuffling (Peterson and Nevalainen 2012; Singhania et al. 2017). The strain improvement processes is one of the most exploited techniques for the enhanced production for different enzymes and have evolved greatly with time. The cloning of enzyme encoding genes in homologous and heterologous hosts has been attempted. *E.coli* is the most favored organism for expression as it has several advantages over other expression system such as yeast or plants. Mandels et al. (1971) had selected a mutant strain *Trichoderma viride* QM6a, from over 100 *Trichoderma* wild strains for enhanced cellulase production. In order to utilize xylose directly, D-xylose-utilizing pathway controlling genes was added to the recombinant *Saccharomyces cerevisiae* HZ3001 (Sun et al. 2012). Similarly Davidi et al. (2016) designed a dockerin-fused variant laccase from the aerobic bacterium *Thermobifida fusca*. The strain improvement processes is one of the most exploited techniques for the enhanced production for different enzymes and have evolved greatly with time.

11.7 Applications of Lignocelluloytic Enzyme in the Bio-Based Economy

LCB has a wide spectrum of applications in various industries. Traditionally, they are applied in food and brewery production, animal feed processing, detergent production, laundry, textile processing and paper pulp manufacturing. Due to the crisis in sustainable supply of fossil fuel, production of biofuels and chemicals from renewable resources are expected to increase rapidly in the foreseeable future. In biorefinery, generation of biofuels and value-added chemicals from renewable LCB

have gained much attention worldwide (Kumar et al. 2020). For utilization of biomass, their depolymerization in order to generate fermentable sugars is one of the basics requirements. Several chemical and physical pre-treatment methods have been suggested; however they are energy intensive and generate several pollutants. Therefore, biological pre-treatment or enzymatic hydrolysis have gained impetuous due to its environmental friendly nature and also has several advantages such as specificity over physic-chemical methods (Agbor et al. 2011). The Table 11.4 represents various strains responsible for cellulase, xylanase and laccase production along with various substrate used, enzyme yield and its application.

11.7.1 Recent Development of the Biocatalyst in the Bio-Based Economy

The recent scenario the conversion of biomass to value-added products, and the focus diverting toward greener methods have gained interest among the researchers. The lignocellulolytic enzymes have been greatly investigated for its ability to produce value-added products as shown in Fig. 11.4 and have been discussed as follows.

11.7.2 Bioethanol and Bio-Based Chemicals

Bioethanol generation from amorphous cellulose and cellulose soaked in ionic liquid were analyzed separately using the recombinant strain *S. cerevisiae* and highest ethanol yield was observed (Yanase et al. 2010; Nakashima et al. 2011). Kumar et al. (2018) demonstrated the application of in-house produced cellulase from *Schizophyllum commune* and xylanase from *A oryzae* LC1 in saccharification of wheat straw and rice straw alone and in combination. The in-house produced

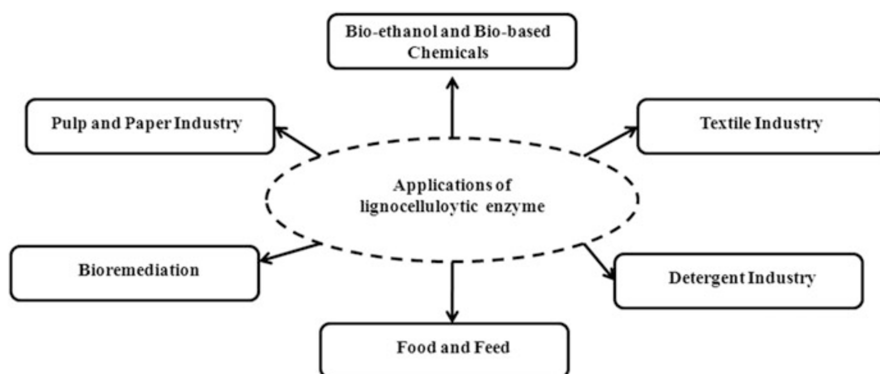

Fig. 11.4 Application of lignocelluloytic enzymes in various fields fitting into the concept of "reduce-reuse-recycle" and contributing toward "circular economy"

cellulase showed results in comparable with the commercial cellulase enzyme Onozuka R-10 from the *Trichoderma viridae.* Bhardwaj et al. (2018) used partially purified xylanase for generating xylooligosaccharides which included xylobiose, xylotriose and xylotetraose by the direct enzymatic hydrolysis of untreated sugar-cane bagasse, wheat straw, and wheat bran. Xylan fermenting thermophilic *Clostridium* strain was used for direct conversion of LCB to bioethanol production. The strain showed preferential bioethanol production efficiency when wide ranges of substrates were tested with mixture of cellulose and xylan and untreated rice straw. This strain also presented broad range of primary metabolic end products such as acetate, lactate, and hydrogen substrate spectrum utilizing wide range of substrate spectrum (Singh et al. 2018b). Similarly laccase from *Lentinus squarrosulus* MR13 resulted in lignin removal from *Saccharum spontaneum (*karn grass) and subsequently resulting enhanced sugar yield (Rajak and Banerjee 2016).

Bhardwaj et al. (2020) demonstrated the synergistic action of laccase, cellulase and xylanase in generation of ethanol using single pot culture system performing simultaneous delignification, saccharification and fermentation. LCB is a sustainable platform for the production of the bio-based chemicals and polymer (Isikgor and Becer 2015; Kumar and Verma 2020b; Agrawal and Verma 2020b). The C5 and C6 carbon generating as a result of hydrolysis of xylanase and cellulase can be used for production of1,4-diacid, 5-HMF, 2,5-FDCA, 3-HPA, aspartic acid, glutamic acid, glucaric acid, itaconic acid, Glycerol, sorbitol, 3-hydroxybutyrolactone, Lactic acid, ABE, xylose-furfural-arabinitol, and levulinic acid platform chemicals.

11.7.3 Textile Industry

The specificity of enzyme has led to development of enzyme as important biochemical in textile industry. The enzymes are highly specific, nontoxic and therefore enzymes have replaced the intensive conventional chemical processes in textile processing. The cellulases and xylanases can effectively remove the hairiness of the cellulose thread with less weight loss, impart brightness and help in giving textile a smooth texture and glossy appearance. Acidic cellulases obtained from the *T. reesei* (Kuhad et al. 2011), neutral cellulases *H. insolens* (Anish et al. 2007) xylanases from *Bacillus pumilu*s (Singh et al. 2018a; Battan et al. 2012)*,* and laccase from *Pleurotus ostreatus and Trametes versicolor* (Anish et al. 2007; Mojsov 2014) are widely used in the textile processing industries.

11.7.4 Detergent Industry

The detergents have several applications such as laundering, dishwashing and cleaning (Schäfer et al. 2005). Traditional chemical detergents have strong bleaching chemicals and phosphates and it adversely affect to the environment and the user (Olsen and Falholt 1998). Due to this drawback, development of detergents for industrial application using enzymes is one of the major research areas today. The

enzymes help in removal of proteins starch, fats oil stains and due to its hydrolytic properties increases effectiveness of detergents (Kirk et al. 2002; Hasan et al. 2010). Different hydrolyzing enzymes such as lipases, proteases, cellulases, pectinase, xylanases and laccases are used in different detergent composition for efficient cleaning and fabric care (Kuhad et al. 2011; Li et al. 2012). Apart from removing stains these enzymes cleaves off damaged cotton fabric and maintain color, whiteness and fabric smoothness. Different alkaline cellulases enzymes are used in detergents which are capable of passing through the inter fibril spaces of the cotton and help in efficient stain removal (Juturu and Wu 2014).

11.7.5 Food and Feed

At present, these so called wastes are judiciously utilized to produce valuable products such as enzymes, sugars, biofuels, chemicals, cheap energy sources for fermentation, improved animal feeds, human nutrients and intermediates compounds for generating value-added chemicals (Kuhad et al. 2011). The enzymes have huge applicability and food in food industry as well α-amylases and xylanases have been used for maintaining the softness and elasticity of bread. Cellulase and laccase has been effectively used for the clarification of juice where it catalyzes the cross-linking of polyphenols, thereby resulting in an easy removal of polyphenols by the help of filtration. It has also been used for flavor enhancement in beer. It is possible that better functional understanding of these enzyme classes will broaden its applications within the food industry. The use of enzyme mixtures containing pectinases, cellulases, and hemicellulases can also be used for improved extraction of olive oil as well.

The enzymes have been effectively used as feed additives as well; xylanases and β-glucanases have been used in cereal-based feed for monogastric animal's (Kirk et al. 2002). The use of pre-treatment of agricultural silage and grain feed by the combination of cellulases or xylanases has helped improve its nutritional value (Godfrey and West 1996) eliminate anti-nutritional factors; degrade certain feed constituents which would help improve its overall nutritional value for its effective use (Choct 2006; Juturu and Wu 2014; Vasconcellos et al. 2015).

11.7.6 Bioremediation

In the wastewater processing sector cellulase enzyme is employed in the bioremediation of the ink released during recycling of paper in pulp and paper industry (Karigar and Rao 2011). Thermo-alkaline tolerant cellulases are new favorites of the scientist working in the area biofuel production and waste management's (Annamalai et al. 2016; Khan et al. 2016) *Humicola* species cellulase is highly adaptive to the harsh environment so it is used in bioremediation of wastewater generated from detergents and washing powder industries (Imran et al. 2016). Laccase also play crucial role in decolorization of azo dyes by oxidizing the aromatic

ring and transform these azo dyes into less harmful by-products (Verma and Madamwar 2005). Agrawal et al. (2020a) and Agrawal and Verma (2020a) demonstrated the white laccase obtained from *Myrothecium verrucaria* ITCC-8447 has resulted in complete removal of hazardous chemical i.e., phenol and resorcinol. Similarly the same group has also reported that yellow laccase obtained from *Stropharia* sp. ITCC 8422 was efficient in degrading anthraquinone violet R and alizarin cyanine green dye (Agrawal and Verma 2019a,b; Agrawal et al. 2020b).

The Commercial laccase obtained from genetically modified *Aspergillus oryzae* effectively treated meat industry wastewater resulting in reduction of COD and color under optimum conditions (Thirugnanasambandham and Sivakumar 2015). Peroxidases has shown capability to treat wide spectra of aromatic compounds such as aromatic compounds (Chen et al. 2014), anilines (Nakamoto and Machida 1992), aromatic dyes (Bhunia et al. 2001), poly-carbonated biphenyls (Köller et al. 2000), poly-aromatic hydrocarbons (Baborová et al. 2006), and phenols (Bayramouglu and Arica 2008), along with different pollutant of industrial contamination (Cheng et al. 2006). These enzymatic biotransformation serve dual purpose in waste management i.e., treatment of waste and generating the several essential chemicals and intermediates.

11.7.7 Pulp and Paper Industry

The utilization of chemicals in the paper and pulp industry is very high, thus the environmental concern has now replaced the chemical based method to the bio-based methods. The use of enzymes has been an effective and promising tool in the paper and pulp industry. Cellulases individually, or in combination with xylanases, can be used for the deinking of various types of paper wastes (Kumar et al. 2018). Maximum applications involving the use of cellulases and hemicellulases leads to the release of ink from the fiber surface by the partial hydrolysis of carbohydrate molecules (Kuhad et al. 2011; Juturu and Wu 2014; Kumar et al. 2018). Similarly, laccase in the paper and pulp industry plays a role in the delignification and brightening of the pulp but can also remove the lipophilic extractives responsible for pitch deposition from both wood and non-wood paper pulps. It can also improve the physical, chemical, as well as mechanical properties of pulp either by forming reactive radicals with lignin or by functionalizing lignocellulosic fibers. Further it also targets the colored and toxic compounds which are released as effluents from various paper and pulp industries rendering them nontoxic by the polymerization and depolymerization reactions (Virk et al. 2012).

11.8 Overcoming the Limitations and Future Prospects Toward Making "Circular Bio-Economy" a Reality

The major challenges toward making circular based bio-economy a reality through feasible solutions are mentioned below:

1. Selection of suitable strains for the lignocellulolytic enzyme production which can be used in synchronization for biomass deconstruction and development of low cost enzyme production process and effective downstream production technologies.
2. Development of biological and environmental friendly pre-treatment technology for minimizing the natural recalcitrance of LCB.
3. Selection of promising strains or development of suitable technology which can be employed for conversion of lignin, xylan, and cellulose to several reactions intermediated for economically important compounds.
4. Supply chain mechanisms of the wastes collection from field to industries and the development of decentralized system for continuous supply of the biomass to the bio-based industries.

These are the major points may be taken into consideration in order to make concept of circular bio-economy a reality in the future (Kumar and Verma 2020a). In this participation of governments, different stack holders, scientist, economist and engineers will play a pivotal role. The cooperation and synergy among these institutions will lead to a sustainable system of the bio-based economy where recycling and waste reduction is reality.

11.9 Conclusion

The application of lignocellulolytic in biomass deconstruction for generation of sugars and value-added products have to a parallel economy where waste materials such as agricultural residues which were earlier considered as waste are now part of the bio-based economy also known as circular bio-economy. The conversion of hemicelluloytic content of LCB by cellulase and xylanase to reducing sugar which can further be converted to bioethanol can act as green substitute to fossil fuels. Xylanase can help in direct conversion of xylan to different xylooligosaccharides which can be used as dietary supplements and essential pharmaceutical The removal of lignin using laccase can enable the better utilization of the agro-residues and contribute toward green environment and circular economy as well. This parallel bio-based economy where the concept of reduce-reuse-recycle is applied on reutilization of waste material, the concept of circular bio-economy evolves mostly around the LCB and these hydrolyzing lignocellulolytic enzymes. The combination of both the concepts by efficient utilization of wastes and technologies can help achieve the goal of "reduce-reuse-recycle" and contribute toward "circular economy" as well.

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Thermostable Enzymes from *Clostridium thermocellum* 12

Abhijeet Thakur, Kedar Sharma, Ruchi Mutreja, and Arun Goyal

Abstract

The production of bioenergy from wastes attracts worldwide attention to overcome energy crisis and increasing pollution (Thakur et al., Microbial fermentation and enzyme technology, Taylor and Francis Group, Boca Raton, FL, 257–268, 2020). Lignocellulosic biomass can serve as an alternative source for bioenergy production. Thermostable enzymes can hydrolyze the lignocellulosic biomass and produce reducing sugars, which can be fermented to produce bioethanol by using fermenting microbes. *Clostridium thermocellum* is a gram-positive, anaerobic and rod-shaped, thermophilic microorganism having great potential applications. It can directly transform lignocellulosic biomass into valuable products such as acetate, ethanol, formate, and lactate. *Clostridium thermocellum* expresses a multi-enzyme complex bound to scaffoldin proteins called cellulosome that contains cellulolytic, hemicellulolytic, and other carbohydrate degrading enzymes. The thermophilic enzymes possess wide applications in several industries for producing sustainable green products. This chapter evaluates the production and properties of recombinant thermostable cellulases, hemicellulases, and pectinases from *C. thermocellum,* their structure, and applications in different industrial processes.

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Keywords

Lignocellulosic biomass · Thermostable enzymes · Biofuels · Consolidated bioprocessing · Oligosaccharides

12.1 Introduction

Microorganisms available in our ecosystem produce potential plant cell wall hydrolyzing enzymes. These enzymes are of great interest and have demonstrated potential uses in different industries such as pulp and paper, detergent, textile, leather, chemical, biofuel, animal feed, food and beverages, pharmaceutical, and cosmetic industries. The increase in demand for plant cell wall degrading enzymes has forced exploration of newer enzymes with improved characteristics like thermostability, pH stability, catalytic efficiency, and production yield. To accomplish the industrial needs, thermophilic bacterial strains have been explored owing to their ability to produce the thermostable enzymes having high catalytic efficiency.

Clostridium thermocellum is a gram-positive, rod-shaped, and anaerobic thermophilic bacterium first reported in 1926 (Viljoen et al. 1926). Recently, *Clostridium thermocellum* is renamed as *Hungateiclostridium thermocellum*. The initial characterization showed that it grows at higher temperatures, between 50 and 68 °C and between pH, 6.2 and 7.7 on substrates such as cellulose, cellobiose, or hemicelluloses and produce acetic acid, succinic acid, formic acid, lactic acid (McBee 1954), CO_2, H_2, and ethanol (Freier et al. 1988). *C. thermocellum* showed the ethanol tolerance of 5 g/L (Herrero and Gomez 1980) because of its high lipid content of the cell wall. Total lipid content in *C. thermocellum* is 0.82 mg/g of dry cell weight (Timmons et al. 2009). The optimum pH 6.7–7 and optimum temperature of 55 °C were reported for the growth of *C. thermocellum* (Freier et al. 1988). The bacterium can be cultured with the maximum growth rate in batch (0.10/h) or continuous mode (0.16/h) under its optimized growth conditions (Lynd et al. 1989). *C. thermocellum* cells, when grown on a cellulosic substrate, forms monolayer biofilms and align themselves along with the cellulosic fiber for its hydrolysis (Dumitrache et al. 2013). With the increasing demand for biofuel, *C. thermocellum* is given attention because of its performance and efficiency for its use in consolidated bioprocessing applications (Olson et al. 2015). *C. thermocellum* releases a multienzyme complex called cellulosome on its outer surface (Bayer et al. 1985). Cellulosomes are crucial for efficient hydrolysis of crystalline cellulose and complex carbohydrates.

12.2 Cellulosomal Structure of *C. thermocellum*

C. thermocellum releases a multienzyme complex extracellularly, which is known as the cellulosome. These are of molecular size more than 2000 kDa having a diameter of approximately, 18 nm (Uversky and Kataeva 2006). The main body of

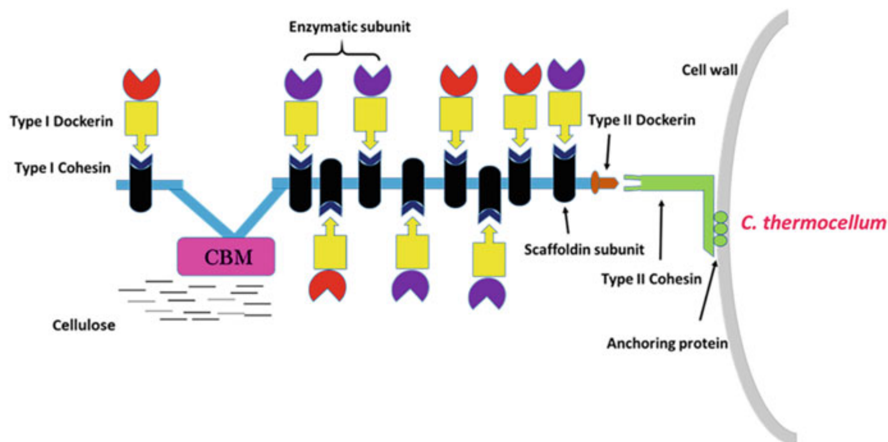

Fig. 12.1 Structure of cellulosomal complex from *C. thermocellum*

cellulosome comprises a non-catalytic scaffoldin protein called cipA (Dror et al. 2003) as shown in Fig. 12.1. cipA contains 9, type I cohesin domains attached to 9, type I dockerins, each having a catalytic subunit, connected through a peptide linker. cipA is linked to the type II cohesin via type II dockerin and the type II dockerin is bound to the bacterial cell wall through a non-catalytic anchoring protein (Dror et al. 2003) as shown in Fig. 12.1. Additionally, cipA possesses a carbohydrate-binding module (CBM), through which the cellulosome adheres to carbohydrate substrates (Gilbert 2007). CBMs are also found associated with the catalytic subunits. The CBM associated with cipA in the cellulosome binds more tightly to the substrate than the CBM associated with the catalytic subunit. The cohesin and dockerin interactions are primarily hydrophobic as revealed by the structural analysis using crystallographic studies (Carvalho et al. 2003) and by molecular simulation studies (Xu et al. 2009). The hybrid scaffoldins containing, consecutively two cohesins from different organisms (one cohesin from *C. cellulolyticum* and the other from *C. thermocellum*) connected through a linker peptide of variable lengths were constructed (Borne et al. 2013). They reported that the length of the linker peptide between the two cohesins affects the binding of dockerin to cohesin. The cellulosomal complex plays a pivotal role in the complete breakdown of crystalline cellulose and delignified plant biomass. The cellulosomal hydrolysis of various substrates not only depends on its enzymatic diversity but also on its structure (Hirano et al. 2016). Cellulosomes contain more than 20 enzymes such as cellulases, hemicellulases, pectinases, esterases, chitinases, and glycosidases making them capable of degrading the complex lignocellulosic biomass (Wertz and Bédué 2013).

C. thermocellum contains enzymes of different classes, which includes 78 gene sequences of glycosidase hydrolase, 37 sequences of the glycosyltransferases, 4 sequences of polysaccharide lyases, 15 sequences of carbohydrate esterases, and 97 sequences of carbohydrate-binding modules (http://www.cazy.org/b507.html).

The 78 sequences of glycoside hydrolase (GH) exist in families 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 16, 18, 22, 23, 25, 26, 28, 30, 32, 34, 35, 39, 42, 43, 44, 48, 50, 51, 53, 54, 62, 74, 81, 84, 94, 124, 126, 130, and 141. Out of 78 sequences, five sequences are not yet assigned to any GH family (http://www.cazy.org/b1479.html). *C. thermocellum* produced cellulolytic, xylanolytic, and pectinolytic enzymes when cultured on crystalline cellulose or plant biomass (Blume et al. 2013). Several cellulases, cellobiohydrolases, glucosidases, xylanases, xylosidases, arabinofuranosidases**,** **mannanase,** pectinases, rhamnogalacturonan lyase, and carbohydrate-binding modules have been reported from *C. thermocellum* and are elaborated in subsequent sections.

12.2.1 Cellulases

The most abundant organic polymer on earth is cellulose. Over half of the carbon in the biosphere is in the form of cellulose. It is also the most critical and integral part of the primary cell wall of angiosperms and gymnosperms (Klemm et al. 2005). Cellulose is a homogenous structure, comprising 100–20,000 D-glucose residues having β-1,4 glycosidic linkages. These are non-branched structures, in which 36–1200 cellulose chains linked together by hydrogen bonds and van der Waals force, forming a well-arranged crystalline structure called microfibrils (Zhang and Lynd 2004). Cellulases hydrolyzes the β-1,4-glycosidic linkage between glucose residues of cellulose. Different kinds of cellulases such as endoglucanase, cellobiohydrolase, and glucosidase are required for the complete enzymatic degradation of cellulose (Lynd et al. 2005). Commonly, the free cellulase consists of a carbohydrate-binding module (CBM) at the C-terminal joined by a short linker region to the catalytic domain at the N-terminal (Gilbert 2007). Cellulase is used in several industrial processes. In the textile industry, it is used for the biopolishing of fabrics and in detergents, it is used for improving the softness and brightness of fabric (Hill et al. 2006). It is also used for de-inking of paper in the paper industry (Qin et al. 2000). Cellulases can be used in animal feed for enhancing the nutrition value and digestibility (Dhiman et al. 2002). In the biofuel industry, the cellulases play a central role in saccharification of pretreated biomass for biofuel or any other value-added chemical production (Lynd et al. 2005). All these industries demand thermostable cellulases.

12.2.1.1 Classification of Cellulases

Cellulases are classified based on their mode of action. Several types of cellulases have been described (Sadhu and Maiti 2013). The cellulolytic enzymes with different activities or specificities are shown in Table 12.1. The cellulosic portion of plant biomass cannot be hydrolyzed by endoglucanases alone. Endoglucanase acts on amorphous site of cellulose polysaccharide and produces cello-oligosaccharides of various lengths. Cellobiohydrolase acts on reducing or non-reducing end of cellulose and releases glucose or cellobiose. Cellodextrinase hydrolyzes cello-oligosaccharides and releases cellobiose, whereas, it is inactive against the

Table 12.1 Types of cellulolytic enzymes and their substrate specificity

Activity	Substrate	EC number	Family
Endoglucanase or glucanohydrolase	Cellulose	3.2.1.4	5, 8, 9, 26, 44, 48, 74, 124
Exoglucanase or cellobiohydrolase	Cellulose	3.2.1.91	5, 9, 48
Exoglucanases or cellodextrinases	Cello-oligosaccharides	3.2.1.74	1, 3, 5, 9
β-Glucosidase	Cellobiose and cellodextrin	3.2.1.21	1, 2, 3, 5, 9, 30, 39
Cellobiose phosphorylase	Cellobiose	2.4.1.20	94
Cellodextrin phosphorylase	Cellodextrin	2.4.1.49	94
Cellobiose epimerase	Cellobiose	5.1.3.11	

polysaccharide. β-glucosidase acts on cellodextrins and cellobiose and releases glucose, but it is unable to hydrolyze the polysaccharide, cellulose. Cellobiose phosphorylase causes reversible phosphorolytic cleavage of cellobiose. Cellodextrin phosphorylase was found in cells of *C. thermocellum* that catalyzes the reversible phosphorolytic cleavage of cellodextrins (Sheth and Alexander 1969).

12.2.2 Hemicellulases

The most abundant polysaccharide after cellulose present in nature is hemicellulose. Unlike cellulose, the hemicellulose is heterogeneous. Hemicellulose accounts for about 20–35% of lignocellulosic biomass. Hemicelluloses include both pentose sugars (arabinose, xylose) containing polysaccharides such as xylan, arabinoxylan, glucuronoxylan, arabinoglucuronoxylan, arabinan, and hexose sugars (galactose, glucose, and mannose) containing such as galactan, arabinogalactan, mannan, glucomannan, galactomannan, and xyloglucan. The softwoods contains hemicellulose composed mainly of glucomannans whereas, the hardwoods, contains mainly xylan (McMillan 1993). Xylan is composed of xylose backbone linked via β-1,4 glycosidic bond with numerous substitutions. The side chains attached to xylose residues present in the xylose backbone are acetic acid, *p*-coumaric acid, ferulic acid, glucuronic acid, and arabinose (Thakur et al. 2019). Based on substitution occurring in the xylan backbone it is categorized as linear homoxylan, arabinoxylan, glucuronoxylan, or glucuronoarabinoxylan. Heterogeneous structure of hemicellulose hinders its enzymatic hydrolysis. Plant biomass contains a significant amount of xylan and for its complete hydrolysis, several enzymes are required. The main chain hydrolysis of xylan is brought about by endoxylanase and xylosidase whereas, several enzymes like ferulic acid esterase, acetyl xylan esterase, *p*-coumaric esterase, arabinofuranosidase, and glucuronidase act synergistically and remove the side chain branching, thus helping in the complete degradation of plant biomass. Mannans are the most abundant hemicellulosic polysaccharide after xylan. Mannan

backbone is composed of mannose residues linked through β-1,4 mannosidic bonds. The side chains of mannan are substituted with glucose and/or galactose and are classified as glucomannan, galactomannan, and galactoglucomannan (Singh et al. 2018b). The complete breakdown of mannans requires, the main chain hydrolyzing enzymes such as β-mannanase (EC 3.2.1.78), β-mannosidase (EC 3.2.1.25) and β-glucosidase (EC 3.2.1.21) as well as side chains removing enzymes such as α-galactosidase (EC 3.2.2.22) and acetyl mannan esterase (EC 3.1.1.6) (Sharma et al. 2018). Hemicellulases are widely used in different industrial processes e.g., biobleaching of pulp, production of bioethanol and value-added chemicals, production of xylo-, arabinoxylo-, Manno-oligosaccharides, in the baking industry as a food additive, in the feed industry to enhance digestibility and in laundry detergent as an additive (Kulkarni et al. 1999).

12.2.2.1 Classification of Hemicellulases

The hemicellulolytic enzymes are classified based on their substrate specificities (Table 12.2). Endo-xylanase hydrolyzes β-1,4 glycosidic bond between two xylose residues of the xylan backbone and releases xylo-oligosaccharides of various degrees of polymerization. Exo-xylanase cleaves xylo-oligosaccharide, to produce xylobiose. This xylobiose acts as the substrate for β-xylosidase and releases two molecules of xylose. α-Glucuronidase and α-arabinofuranosidase release glucuronic acid from glucuronoxylans and arabinose from arabinoxylan, respectively. β-Mannanase catalyzes hemicelluloses containing mannan and releases β-1,4-manno-oligomers, which are further hydrolyzed by β-mannosidases to mannose. Acetyl xylan-, *p*-coumaric acid-, and ferulic acid esterase hydrolyze their respective ester bonds present in xylan.

Table 12.2 Types of hemicellulolytic enzymes and their substrate specificity

Activity	Substrate	EC number	Family
Endo-xylanase	Xylan	3.2.1.8	3, 5, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51, 62, 98, 141
Exo-Xylanase	Xylan	3.2.1.156	8
β-Xylosidase	Xylo-oligosaccharides	3.2.1.37	1, 3, 5, 30, 39, 43, 51, 52, 54, 116, 120
α-Glucuronidase	Glucuronoxylan	3.2.1.139	4, 67
α-Arabinofuranosidase	Arabinoxylan	3.2.1.55	2, 3, 10, 43, 51, 54, 62
Endo-α-1,5-arabinanase	Arabinan	3.2.1.99	43
Acetyl xylan esterase	Xylan	3.1.1.72	1, 2, 3, 4, 5, 6, 7, 12, 15
Ferulic acid esterase/*ρ*-Coumaric acid esterase	Methyl ferulate/methyl *p*-coumarate	3.1.1.73	1
β-Mannanases	β-Mannan	3.2.1.78	5, 9, 26, 44, 113, 134
Exo-β-1,4-mannosidase	β-1,4-Mannooligomers	3.2.1.25	1, 2, 5
Endo-galactanase	β-D-galactan	3.2.1.89	53
β-Galactosidase	Galactan	3.2.1.23	1, 2, 35, 42, 59, 147

12.2.3 Pectinase

Pectin-degrading enzymes or pectinolytic enzymes or pectinases hydrolyze the pectin. Pectin is found in the middle lamella as well as also in the primary cell wall of plants (Scheller et al. 2007). Pectin is composed of homogalacturonan (HG), rhamnogalacturonan type-I (RG-I), and rhamnogalacturonan type-II (RG-II) cross-linked to each other in an unknown manner (Atmodjo et al. 2013). The most abundant pectin is HG which is a linear homopolymer composed of α-D-galacturonic acid and may be methylated at C6 position while the RG-I and RG-II are branched structures of pectin. RG-I contains L-rhamnopyranosyl (αL-Rhap) and D-galactopyranosyluronic acid (D-GalpA) residues alternately, in the main chain. The αL-Rhap residue present in the backbone may be substituted with α-L-arabinofuranose and β-D-galactopyranose residues (Dhillon et al. 2016). Rhamnogalacturonan II comprises at least eight 1,4-linked D-galactopyranosyluronic acid residues, which are substituted with 12 other different monosaccharide residues (O'Neill et al. 2004). Pectinases widely cover the market by contributing approximately, 25% share in the global sales of food enzymes, thus can be considered as monumental for the industrial sector (Patil and Dayanand 2006). The sources of commercial pectinases are fungi (Singh et al. 1999), yeast (Barnby et al. 1990) as well as bacteria (Kashyap et al. 2000). *C. thermocellum* has been widely explored for the production of pectinases owing to the unique properties offered by the thermophiles. The pectinases have been used in different industries such as food (Kashyap et al. 2001), paper and pulp and textile (Kuhad et al. 1997), biomass processing (Volynets et al. 2017), tea or coffee processing (Jayani et al. 2005), vegetable oil extraction (Kashyap et al. 2001) and animal feed and wine processing (Sieiro et al. 2012). The pectinase classification, its production, and industrial applications have been discussed in the subsequent sections.

12.2.3.1 Classification of Pectinases

Pectinases can be of two types (1) de-esterifying and (2) depolymerizing enzymes depending upon the type of linkage. Pectin methylesterase (PME) and pectin acetylesterase (PAE) are de-esterifying enzymes and they hydrolyze the pectin by removing methoxyl groups and acetyl groups, respectively, from galacturonate units of pectin (Dubey et al. 2016). PME and PAE both exist in the carbohydrate esterase (CE) families CE8 and CE12 according to the CAZy system of classification (Lombard et al. 2014). The depolymerizing enzymes can be glycoside hydrolases (GH) and polysaccharide lyases (PL) which break the α-1,4 linkages present in polygalacturonic acid (PGA) by hydrolysis (Tapre and Jain 2014) and β-elimination (Garg et al. 2016), respectively. Pectinolytic enzymes are the broad class of enzymes, which are classified in GHs, PLs, and CEs based on the type of bond they cleave. These enzymes have different substrate specificities and activities as shown in Table 12.3.

Table 12.3 Pectinolytic enzymes and their substrate specificity

Activity	Substrate	EC number	Family
Polygalacturonase	Polygalactouronic acid	3.2.1.15	28
Exo-polygalacturonase	Polygalactouronic acid	3.2.1.67	4, 28
Pectin methylesterase	Pectin with methylation	3.1.1.11	8
Pectin acetylesterase	Pectin with acetylation	3.1.1.6	1, 3, 4, 16
Pectate lyase	Polygalactouronic acid	4.2.2.2	1, 2, 3, 9, 10
Exo-pectate lyase	Polygalactouronic acid	4.2.2.9	1, 2, 9
Pectin lyase	Polygalactouronic acid	4.2.2.10	1
Rhamnogalacturonan I rhamnohydrolase	Rhamnogalacturonan	3.2.1.174	78, 126
Rhamnogalacturonan I galactohydrolase	Rhamnogalacturonan	3.2.1.173	28
Rhamnogalacturonan I endo-hydrolase	Rhamnogalacturonan	3.2.1.171	28
Rhamnogalacturonan lyase	Rhamnogalacturonan	4.2.2.23	4, 9, 11
Exo-rhamnogalacturonan lyase	Rhamnogalacturonan I oligosaccharides	4.2.2.24	11, 26
Unsaturated rahmnogalactouronyl hydrolase	Rhamnogalacturonan	3.2.1.172	105

12.3 Production of Carbohydrate-Active Enzymes from *C. thermocellum*

Various organisms belonging to actinomycetes, bacteria, and fungi are known to produce carbohydrate-active enzymes (Juturu and Wu 2014). During the growth on the cellulosic substrate, the organism produces cellulases. The extracellular cellulases from fungi and bacteria are released into the medium which is easier to recover and purify. Various thermostable carbohydrate-active enzymes elaborated by *C. thermocellum* are listed in Table 12.4. *C. thermocellum* B8 isolated from the rumen of goat showed the ability to grow on sugarcane straw and cotton waste (Leitão et al. 2017). The microorganism gave significant production of cellulase and xylanase after 48 h at 60 °C and pH 7.0. Nowadays the genes encoding cellulases are cloned and hyper-expressed in the fast-growing host like *Escherichia coli* for their higher production. Bacterial strains are more capable than fungal in tolerating environmental stress like temperature and pH. The recombinant thermostable cellulase (Cel9R) of family 9 GH (Zverlov et al. 2005b) and GH5 (Bharali et al. 2005) from *C. thermocellum* were cloned, overexpressed, and characterized (Table 12.1). Cel9R showed activity against carboxymethylcellulose (CMC), barley β-glucan, phosphoric acid swollen cellulose (PASC), and *p*NP-glucosides. Whereas GH5 showed activity against β-glucan, CMC, lichenan, avicel, and also oat spelt xylan. A functional subgenomic study of *C. thermocellum* genome showed 71 celluosomal

Table 12.4 Various plant cell wall degrading enzymes from *C. thermocellum*

Enzyme	Mw kDa	Opt temp (°C)	Opt pH	Substrate	References
Cellulase (GH5)	35	50	4.2	CMC, β-glucan and oat spelt xylan	Bharali et al. (2005)
Cellulase (Cel9R)	75	78	6	PASC, CMC, β-glucan, *p*-NP-glucosides	Zverlov et al. (2005a)
Cellulase (Cel48Y)	69	65	7	Amorphous cellulose	Berger et al. (2007)
Cellulase (Cel124A)	30	60	5–7	PASC, CMC, β-glucan	Brás et al. (2011)
Cellobiohydrolase (CelO)	72	65	6.6	β-Glucan, CMC, PASC	Zverlov et al. (2002)
Glucosidase (*Ht*GH1)	52	65	6	*p*-NP-glucopyranoside, cellobiose, *p*-NP-galactopyranoside	Sharma et al. (2019)
Laminarinase (Lam81)	82	75	7	Laminarin, Curdlan	Kumar et al. (2018)
Lichenase (*Ct*Lic26A)	30	60	7	Lichenan	Taylor et al. (2005)
Mannanase (Man26A)	70	65	6.5	Carob galactomannan, ivory-nut mannan	Halstead et al. (1999)
Mannanase (Man26B)	38	50	6.5	Carob galactomannan, locust bean galactomannan	Ghosh et al. (2013)
Glucuronoxylanase (*Ct*XynGH30)	60	70	6	Beechwood xylan and 4-O-methyl glucuronoxylan	Verma and Goyal (2016)
Arabinofuranosidase (AxB8)	50	50	5–6	*p*NP-β-D-xylopyranoside, *p*NP-α-L-arabinofuranoside	de Camargo et al. (2018)
Arabinofuranosidase (*Ct*GH43)	34	50	5.4	Rye arabinoxylan, oat spelt xylan, *p*NP-α-L-arabinofuranoside	Ahmed et al. (2013)
Rhamnogalcturonan Lyase (*Ct*RGL)	64	60	8.5	Rhamnogalacturonan, galactan, polygalacturonic acid, and pectin	Dhillon et al. (2016)
Pectate Lyase (PL1A) Pectate Lyase (PL1B) Pectate Lyase (PL9)	34 40 32	50 50 60	8.5 9.8 8.5	Polygalacturonic acid, pectin (citrus) (25% methylation)	Chakraborty et al. (2015)
Pectin methylesterase (*Ct*PME)	35	50	8.5	Citrus pectin (>85% methylation)	Rajulapati and Goyal (2017)

genes and one-third of these have the capacity for cellulose degradation (Zverlov et al. 2005a).

C. thermocellum cellulosome also contains several hemicellulolytic enzymes (Zverlov et al. 2005a). *C. thermocellum* can grow on cellobiose only without utilizing any hemicellulosic derived sugar (Shallom and Shoham 2003). *C. thermocellum* when grown on cellulose, the expression for cellulase (GH9) was higher, while the expression of cellulases GH5 and GH8 and hemicellulases was higher when grown on cellobiose (Gold and Martin 2007). Out of 81 cellulosomal genes reported for *C. thermocellum*, 21 encode for hemicellulases (Krauss et al. 2012). Transcriptomic and proteomic study of *C. thermocellum* when grown on sugarcane bagasse showed the expression of arabinofuranosidase (axb8) and β-1,3-galactosidase (de Camargo et al. 2018). For higher production of hemicellulases, their genes can be cloned in a suitable vector and transformed in fast-growing *E. coli* host strain for hyper-expression. Mannanase of family 26 GH (Man26A) (Halstead et al. 1999) and Man26B (Ghosh et al. 2013), arabinofuranosidase of family 43 (*Ct*GH43) (Ahmed et al. 2013), xyloglucan of family 74 (Xgh74A) (Martinez-Fleites et al. 2006) and glucuronoxylan endo-β-1,4-xylanase of family 30 (*Ct*XynGH30) (Verma and Goyal 2016) were cloned, hyper-expressed, purified, and biochemically characterized.

The pectinolytic activity of *C. thermocellum* was also reported (Spinnler et al. 1986). Three different pectin-degrading enzymes (PL1A, PL1B, and PL9) from *C. thermocellum* were identified, cloned, and characterized (Chakraborty et al. 2015). These pectinases displayed activity against PGA and pectins. Pectic substrates are highly heterogeneous, therefore, their complete breakdown requires several enzymes. In another study, thermostable pectin methylesterase (*Ct*PME) of family 8 carbohydrate esterase from *C. thermocellum* was cloned in pET28a(+) vector and characterized (Rajulapati and Goyal 2017). Multifunctional acetyl xylan esterase (*Ct*CE2) exhibited catalytic activity against acetylated xylan and also displayed non-catalytic carbohydrate-binding function against cellulosic substrates (Montanier et al. 2009). An alkaline rhamnogalacturonan lyase (RG lyase) *Ct*RGL*f*, of family 11 polysaccharide lyase from *C. thermocellum* was also characterized (Dhillon et al. 2016). Pectin acetylesterase from *C. thermocellum,* is available commercially from NZYTech, Lda, Lisbon, Portugal (https://www.nzytech.com/), and creative enzymes, New York, USA (https://www.creative-enzymes.com/). The production of enzymes from *C. thermocellum* has been reported by both solid-state fermentation and submerged fermentation (Couri et al. 2000; Friedrich et al. 1990; Kashyap et al. 2000).

12.4 Application of Thermostable Enzymes from *Clostridium thermocellum*

The carbohydrate-active enzymes from *C. thermocellum* are thermostable modular enzymes as they also contain thermostabilizing domains such as carbohydrate-binding modules associated with the catalytic modules (Fontes et al. 1995). Several

enzymes elaborated by *C. thermocellum* have the potential to hydrolyze biomass, therefore can be used in various other industries. The potential of thermostable carbohydrate-active enzymes from *C. thermocellum* has been also explored in the healthcare and textile industries.

12.4.1 *Clostridium thermocellum* and Its Enzymes in Biofuels

Lignocellulosic bioresources can provide eco-friendly and economical biofuel without competing with food crops. The major hurdle in bioethanol production is the conversion of lignocellulosic biomass to monosaccharides. Various physical (Borah et al. 2016), chemical, physiochemical, thermal (Jamaldheen et al. 2019), microbial, and combined strategies (Singh et al. 2015) have been applied for monomeric sugar production. The recombinant cellulase (C*t*Lic26A-Cel5-CBM11) from *C. thermocellum* was used along with C6 sugar utilizing *Saccharomyces cerevisiae* on acid pretreated *Syzygium cumini* (Jamun), that yielded 1.42 g/L bioethanol (Mutreja et al. 2011). Cellulase (*Ct*GH5) and hemicellulase (*Ct*GH43) from *C. thermocellum* along with the fermenting microorganisms viz. *Candida shehatae* and *Saccharomyces cerevisiae* on steam-exploded *Achnatherum hymenoides* (wild grass) showed the ethanol yield of 23 g/L after scaling up to the bioreactor (Das et al. 2013). The use of pectin-degrading enzyme reduces the enzyme loading for saccharification of lignocellulosic biomass (Xiao and Anderson 2013). In another study, the production of bioethanol using recombinant hemicellulases (acetyl xylan esterase and *Ct*GH43) from *C. thermocellum* and C5 sugar utilizing *Candida shehatae,* at bioreactor scale displayed 6.75 g/L bioethanol from poplar leaf biomass (Gupta et al. 2014).

Consolidated bioprocessing (CBP) is a potential approach for sustainable and economical production of biofuels. In CBP both saccharification and fermentation occur simultaneously, therefore reducing the cost of biofuel production. *C. thermocellum* becomes a popular choice to be used in CBP owing to its potential to saccharify and ferment in a single step (Olson et al. 2012). Several approaches like the mutation of a gene and metabolic engineering of organisms are used to block mixed acid fermentation and higher ethanol production (Olson et al. 2015). Genetic manipulation enhanced ethanol tolerance of *C. thermocellum* to 15–20 g/L (Shao et al. 2011). Approximately, 95% cellulose hydrolysis and 0.30 g ethanol/g crystalline cellulose (Avicel) was achieved by using *C. thermocellum* thorough CBP approach (Singh et al. 2018a). Butanol production from lignocellulosic biomass was also be achieved by the CBP approach (Nakayama et al. 2011). The mixed culture of *C. saccharoperbutylacetonicum* and *C. thermocellum* were grown on crystalline cellulose that gave 7.9 g/L of butanol. The genes involved in isobutanol metabolism were cloned in a vector and transformed in *C. thermocellum* resulting in integration into its genome by single crossing over (Lin et al. 2015). The engineered *C. thermocellum* strain was used for isobutanol production that gave 5.4 g/L yield in 75 h.

12.4.2 Enzymes from *Clostridium thermocellum* in Functional Food and Healthcare

The manno-oligosaccharides (MOS) were produced by hydrolyzing the defatted and pretreated copra meal using endo-mannanase (*Ct*ManT) of family 26 GH from *C. thermocellum* (Ghosh et al. 2015). These MOS displayed prebiotic potential as they promoted the growth of *Bifidobacterium infantis* and *Lactobacillus acidophilus* higher than the standard prebiotic inulin. The MOS also exhibited anticancer potential as they decreased the cell viability of human colon cancer cell line (HT-29) by 60% after 48 h. Therefore, MOS can also be used as food supplement for functional food applications. Pectic oligosaccharides were produced by pectate lyase, a family 1 polysaccharide lyase (PL1B) mediated enzymatic depolymerization of waste peel of *Citrus Limetta.* (Chakraborty et al. 2018). These pectic oligosaccharides displayed inhibitory effect on of human colon cancer cell (HT-29) line by decreasing its cell viability up to 77% by 24 h.

12.4.3 Enzymes from *Clostridium thermocellum* in Textile Processing

The family 1 polysaccharide lyase, a pectate lyase (PL1B) from *Clostridium thermocellum* was immobilized on magnetic nanoparticles (MNPs) for bioscouring of cotton fabric (Chakraborty et al. 2017). The treatment of cotton fabric by PL1B, displayed enhanced wettability, resulting in a reduction in the time of water absorption from 21 min to 15 s. The effect of recombinant rhamnogalacturonan lyase (*Ct*RGL*f*) on degumming of jute fiber and bioscouring of cotton fabric was evaluated (Dhillon et al. 2019). *Ct*RGL*f* displayed the effective removal of waxy compounds from the surface of jute fibers. Similarly, *Ct*RGL*f* treated cotton fabric also exhibited a reduction in water-absorbing time from 40 min to 30 s.

12.5 Conclusion

The thermophilic carbohydrate-active enzymes have several industrial applications such as biofuel, therapeutics, functional food, paper, and pulp and textile, etc. This chapter reports the thermostable cellulosomal enzymes viz. cellulases, hemicellulases, pectinases, and carbohydrate esterases produced by *C. thermocellum* and also their applications. The majority of the carbohydrate-active enzymes from *C. thermocellum* are employed in the biofuel production. However, several clostridial hemicellulases and pectinolytic enzymes have been implicated in therapeutic, food, and textile applications. These thermostable enzymes have immense potential in food industries for fruit juice clarification, baking industry, and for the production of functional food supplements.

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Hot and Cold Bacteria of Sikkim: Biodiversity and Enzymology

13

Sayak Das, Mingma Thundu Sherpa, Ishfaq Nabi Najar, and Nagendra Thakur

Abstract

The obnubilated macrocosm of microbes are influential, herculean, and is an enigma to us “the humans”—supposedly the wisest species to be evolved ever. The insane ability of microorganisms to survive, adapt, and utilize or metabolize, on every possible nook on earth is like a whodunit and supreme mastery. Their hejira from humans and also the ability to “live-out and live within” us hoodwinking our defence system is shrouded and veiled. In, Sikkim, the north-eastern state of India, diversified micro-flora, and fauna thrive in almost the entire landscape. It is a privilege to have both the extreme conditions of life here at Sikkim—HOT and COLD! The glacier hosts psychrophiles and the hot spring incubates thermophiles. Thus, the exploration of extremozymes from both these special ecology is one of our prime research interests. In this chapter, we have discussed briefly about our research findings on bacterial diversity at hot springs and glaciers of Sikkim. Among hot springs, we have discussed our studies from Borong, Dzongu, Polok, Reshi, Yumthang, and Yume Samdung whereas among glaciers, we have focused on Changme Khang, Changme Khangpu, Chumbu, and Kanchengayao. Some potential bacteria as polyextremophiles have also been highlighted.

Keywords

Thermozyme · Cryozyme · Hot spring · Glacier · *Geobacillus* · *Pseudomonas* · Sikkim

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13.1 Introduction

Enzymes—the indispensable constituent of bio-based chemistry are the most sought after biocatalyst in the twenty-first century. Chemical-based catalyst has been in use since time immemorial but owing to various disadvantages microbial based enzymes are in great commercial demand. Microbial enzymes are highly specific, can adjust to temperature variability, and can cause reactions at higher rates in comparison to their chemical-based catalysts (Drauz and Waldmann 2012). *Bacillus* sp. have better advantange than the other microbes as they can release extracellularly their enzymes like protease, amylase, xylanase, lipase, arabinose, etc. that are very crucial as precursor molecules (Joo et al. 2007). Industrial criteria for a good enzyme focus on stability, specificity, and biodegradability (Kumar et al. 2012). Microbial enzymes are used in almost every industry like food and beverages, textile, paper, biofuel, saccharification, etc. (Drauz and Waldmann 2012; Gupta et al. 2003). Enzymes from extreme sources like thermophiles and psychrophiles are more suitable for industrial applications.

Thermophiles are a branch of extremophiles derived from a Greek word "thermotita" which means "heat-loving organisms." Thermophiles are the microbes that usually reside above 40 °C (Horiike et al. 2009). Thermophiles can be distributed into three types depending on their optimum range of temperature—(1) moderate thermophiles have optimum growth temperature around 40 °C–60 °C (e.g., *Clostridium tepidiprofundi*); (2) true thermophiles have optimum growth temperature in the range of 60 °C–70 °C (e.g., *Thermus aquaticus*) and (3) hyperthermophiles have optimum growth temperature above 80 °C (e.g., *Thermus flavus*) (Pikuta et al. 2007). Thermophilic microorganisms have acknowledged a great curiosity in modern days because their proteins and enzymes are not usually denatured at high temperature. Most of the mesophilic enzymes get denatured at around 40 °C and are completely inactivated beyond 60 °C, whereas some thermophilic enzymes show a greater activity at 80 °C and above as well as a greater half-life at these temperatures (Satyanarayana et al. 2005).

Thermophiles are naturally designed in such a way that they can tolerate the extreme environments in which they inhabit. An increase in temperature changes their membrane and protein dynamics to withstand the environmental extreme conditions (Pikuta et al. 2007; Satyanarayana et al. 2005; Tattersall et al. 2012).

A psychrophilic prokaryote is characterized by those organisms which grow optimally below 15 °C temperature, maximum growth at 20 °C, with no growth above 20 °C temperature (Canganella and Wiegel 2011) and thus they can survive cold environments (Dalmaso et al. 2015; Margesin and Miteva 2011). Another term is "psychrotolerant" which optimally grows above 20 °C and can tolerate less than 5 °C (Canganella and Wiegel 2011). True psychrophiles are called "stenopsychrophiles" (Dalmaso et al. 2015). Cold environments include permafrost, arctic, and Antarctic ice, rocks in very cold regions, permanent cold seawater of polar regions, permanently cold marine (−2 °C), freshwater, deep rock aquifers, and all cold-blooded organisms (Canganella and Wiegel 2011; Miteva and Brenchley 2005; Miteva et al. 2004).

Adaptation of the organism at these low temperatures is due to cold shock proteins and RNA chaperons (Dalmaso et al. 2015). These cold shock or cold adaptive proteins first binds with RNA molecule to maintain its single-stranded (ss) conformation and these cold shock domains (Cold shock protein + RNA) then facilitate cold adaptation (Ramana et al. 2000). Other factors involved are secondary cold-active metabolites, enzymes, antifreeze protein and more important membrane fluidity (Casanueva et al. 2010). The higher content of alpha-helix in protein leads to maintain flexibility at low temperature (Madigan et al. 2009). The high content of unsaturated fatty acid in lipid helps to maintain semifluid state in the membrane to adapt in these extreme temperatures (Deming 2009). Less side-chain interaction among proteins also allows enzymes to be functional at minimal kinetic energy (Satyanarayana et al. 2005).

13.2 Hot Springs of Sikkim

The natural groundwater discharge point through which elevated temperate water flows to its environment is called hot spring (Sen et al. 2010). Every hot spring has its definitive characteristics like temperature, pH, and salinity, which governs the ecological niche constitution and characterizes the microbes present in its habitat. Temperature acts as a natural selection criterion that which dictates their morphology and allows the growth of only those microbes which can withstand higher temperature or tolerate these extreme conditions which can vary from 40 °C to 120 °C or above (Kumar et al. 2013; Sharma et al. 2013). The predominant thermal ecologically active regions are geothermally heated soils, hot springs, geysers, fumaroles, and solfatara (Adiguzel et al. 2009; Sen et al. 2010).

In India, the geothermal exploration began in early 1973 by Geological Survey of India and they reported more than 350 hot springs having temperature range varying above 40 °C–100 °C throughout the entire subcontinent region. Based on the tectonic movements, the hot springs of India were categorized into orogenic and non-orogenic (Bisht et al. 2011). Sikkim naturally hosts many hot springs. It is a major tourist attractive state of India where nature is in its juvenile form and a refreshing season greets its visitors. In local languages, these Hot Springs are called as *Tatopani* or *Tsha chu*. *Tatopani* is a Nepali word where "*Tato*" means Hot and "*pani*" means water whereas "*Tsha chu*" is a Tibetan word where "*Tsha*" means Hot and "*chu*" means water. Here at Sikkim, hot springs are sociologically very significant and hold a prime importance (Das et al. 2012a). It is regarded as an elixir and is believed that bathing in it can cure many bone-related diseases and drinking it can also cure gastric problems (Das et al. 2012b). Located at various places Yumthang, Yume Samdung, Tarum, Polok, Borong, Reshi, etc. hot springs are major tourist attractions.

Polok *Tatopani* (Ralang *Tchu*/Ralang *Tsha chuu*/Rabong *Tatopani*) is located at the base of Gangyab, West Sikkim by the banks of the river Rangeet (Thakur et al. 2013). The trail to the *Tatopani* spot is from Polok, South Sikkim but the *Tatopani* and its source is located at Gangyab foothill, West Sikkim just away from the River

Rangit. **Borong *Tatopani*** is located at lower Borong and the ponds are situated at the banks of river Rangit, in West Sikkim. There are three ponds for bathing but it depends on seasons. **Reshi *Tatopani*/***Phur Tsha Chu* (“*Phur*” means bubble in Tibetan language) and is located approximately 25 km from Jorethang to the east of Reshi (Tinkitam) (Sherpa et al. 2013). Hot spring source is located near the bank of river Testa. Separate time table for male and female hot spring goers were provided by *Phur Tsha Chu* committee (5–8 am for men, 8–11 men, 11–2 pm women, and after 2 pm onwards till 5 pm for men). One can feel a strong sulfurous smell from going closer to the hot spring vicinity. **Yumthang *Tatopani*** is located on the base of the mountain across the river Lachung Chu in the town of Lachung. **Dzongu *Tatopani*** is located in the valley of Lower Dzongu, Sikkim. Dzongu is closely associated with three terms—Land of Lepchas, natural hub of medicinal plants, and interaction of nature and culture. A huge pipe has been connected to this bore channel and it was connected to the bathing house where artificially two ponds have been constructed. Ponds are like modern pools for bathing purposes. Two separate bathing ponds are present—one for males and the other for females in separate two rooms. The water is used only for the bathing purpose (Das et al. 2016). **Yume Samdung *Tatopani*** is located in the North Sikkim district at Yume Samdung valley. It is above zero point and is located at the highest altitude. **Takrum *Tatopani*** is located at Lachen valley in the North Sikkim district.

13.3 Glaciers of Sikkim

Glaciers are the delicate and susceptible biomarkers used for climate change estimation. Biotic and abiotic factors in nature can influence their response like glacial length, temperature, glacier mass balance, and snowline. These factors directly or indirectly affluence the climatic response in the ecological niche (Agren 2010). Himalayan glacier retreat has been in reports since 1850 or “Little Ice Age” which led to an increase in atmospheric temperature (Armstrong 2010; Zemp et al. 2008). They approximately are spread over 33,050 sq. km. (Zemp et al. 2008) which corresponds to ~29% glaciers of Central Asia; ~5% ice caps, and other glaciers of the world. During glacier retreat, the soil gets exposed and is succeeded by algal, fungal, and plant biome in the niche (Bajracharya et al. 2007). Chronosequence created by glaciers presents a shift in the energy and biosphere which ultimately affects the ecology and further the dwelling livelihood of habitants (Gurung and Bajracharya 2012).

In the context of Sikkim Himalayas, here the retreat of glaciers has minimal documentation as only a few have been accessible. There have been geological perspective researches on glacial forelands, moraines, snow-cover, etc. (ICIMOD 2009), in the Himalaya but microbial community analysis, and their niche study is sparse. It has been hypothesized that there might be various factors that regulate the microbial niche in the glaciers like aerosol deposits, dust particles, wind velocity, light intensity, altitude, etc. (Zhang et al. 2007). The wet/dry cycles of glaciers also control the glaciation process and this phenomenon can change the glacial flux

dynamics of snow deposition and microbial accumulation or transportation. The bacterial deposits can be found within the ice core sections and they can help in deciphering the microbial response to the local weather conditions at the deposition time (Priscu et al. 2007).

Sikkim hosts many glaciers within its vast geography—Zemu Glacier, Tasha Glacier, Talung Glacier, Jumthul Glacier, Lhonak Galcier, Rathong Glacier, Theukang Glacier, Teesta Glacier, Tenbawa Galcier, Tongshong Glacier, Chuma Glacier, Umaram Glacier, Changsang Glacier, Yulhe Glacier, etc. (Sherpa 2018). But among all of these only two glaciers, Rathong and Zemu have been studied both geologically and microbiologically. These are also of prime importance as they are the source of Rangit and Teesta rivers. Sikkim Himalayas have recently been subjected to high seismic shocks. The earthquakes, cyclones, avalanches, precipitation, etc., cause an impact on the glaciers and may play a role in the rapid melting of glaciers.

Our research work was concentrated on four glaciers of Sikkim—Changme Khangpu (CK), Changme Khang (CKG), Chumbu, and Kanchengayao glaciers. They were selected as they had not been documented ever and had no reports of any study earlier. They were virgin glaciers in terms of their research and glaciological studies. CK and CKG glacier are situated at Sebu valley of Teesta river basin in North Sikkim (Sherpa et al. 2018). CKG glacier is debris-free glacier and CK is a debris cover glacier. Chumbu glacier originated from the south slope of Chumbu peak. Meltwater of these glaciers feeds into Sebu basin which is ultimately merged into Lachung river of North Sikkim. Kanchengayao glacier, on the other hand, is located at Lachen, Thangu valley, North Sikkim, India. Kanchengayao glacier is a debris-free transverse valley glacier. Kanchengayao glacier originated from south slope Mt. Kanchengayao peak, trending north-south face. Meltwater of this feeds Thangu River of Lachen River, Lachen, North Sikkim.

13.4 Biotechnological and Industrial Significance of Extremozymes

13.4.1 Thermophiles and Thermozymes

Biotechnology has clearly changed our lives in many captivating ways which are inexorable. Many of the reactions involved in biotechnological or industrial processes to develop outputs, need to take place on extremes of temperature, pH, pressure, and salinity (Coker 2016). The mesophilic macromolecules can be utilized in these processes, but being temperature susceptible, these macromolecules must be genetically or chemically modified to harvest the products. However, these modifications can be lengthy and cost-effective (Siddiqui et al. 2009). In contrast, nature has fervently provided with suitable alternatives in the form of extremozymes which are present in microorganisms that can bloom in extremes of temperature, pressure, salinity, and pH (Deming and Baross 2001). These naturally thermostable macromolecules are being already used in various industrial processes. Nonetheless,

the chase has additionally filled in the previous quite a while by industry's acknowledgment that the "survival units" controlled by extremophiles can conceivably serve in a variety of uses. The various applications which have made paradigm shifts in the field of biotechnology are the discovery of polymerase from a thermophilic bacterium *Thermus aquaticus* (Ishino and Ishino 2014). Other applications include biofuel production using various thermophilic enzymes (Barnard et al. 2010), thermophilic microorganism used in biomining (Johnson 2014) and carotenoids used in the food and cosmetic industries (Oren 2010).

Enzymes obtained from thermophilic microorganisms have incomparable physiognomies, for example, temperature, pH, and chemical stability. These proteins or enzymes are inherently more stable under extreme environments than those present in their mesophilic analogs (Satyanarayana et al. 2005). Thermal sensitivity has been the foremost problem to the widespread use of enzymes as far as industry is concerned. The benefits of thermozymes are that due to high temperature there is a lesser chance of contamination, improved reaction rates, substrate solubility, and lower viscosity (Joshi and Satyanarayana 2013). From the biotechnological point of view, the thermophiles are the most attractive microbes on earth due to their ability to produce enzymes that can easily catalyze industrial processes at higher temperatures than their corresponding mesophiles. Thus, the ability of thermophilic enzymes to suitably work at high temperature implies many advantages for their applications in industry (Satyanarayana et al. 2005). Thus, these thermostable enzymes held an explicit allure for researchers all over the world. Mesophilic hosts like *E. coli*, *B. subtilis,* and yeasts were successfully cloned by the thermo enzyme encoding genes for retrieving archaeal genes (Deming and Baross 2001).

13.4.1.1 DNA Polymerases

The discovery of natural stability of DNA polymerase at higher temperature led to the introduction of robust PCR method. PCR is generally used to amplify the nucleic acid sequences, which in turn has found several applications directly or indirectly in biotechnology, genetic engineering, medical, pharmaceutical, and many other fields. Besides, PCR, DNA polymerase enzyme is exploited in DNA cloning, DNA sequencing, whole genome amplification (WGA), single nucleotide polymorphism (SNP) detection, molecular diagnostics, and synthetic biology (Gardner and Kelman 2014). There are many steadfast DNA polymerases used in the above techniques such as *Taq*, *Pfu,* and *Vent* which were isolated from thermophiles *Thermus aquaticus*, *Pyrococcus furiosus*, and *Thermococcus litoralis,* respectively (Satyanarayana et al. 2005). *Taq* polymerase was industrialized and $2 billion royalty was earned by PCR rights holders during its patent (Fore et al. 2006). The distinguishing characteristics of each DNA polymerase may encourage the impending advancement of exclusive reagents and thus the exploration of a new type of DNA polymerase will be of prime foremost emphases in future studies (Ishino and Ishino 2014).

13.4.1.2 Biofuel Production

Alternative support to replenish the nonrenewable resources such as fossil fuels, there is a strenuous effort to produce analogous fuels using biomassess such as corn, sugar cane, and wheat etc. These are known as the biofuels (Coker 2016). The classification of the biofuels can be carried out based on the consumption of source and on the basis of product formed. Thus, based on the source utilization, biofuels are known as first-generation biofuels which can be derived from easily hydrolyzing sugars like starches or oils; or second-generation biofuels which are generated from not easily hydrolyzed such as lignocellulosic material. However, on the basis of end product, biofuels can be classified as bioethanol, biodiesel, bio-butanol, hydrogen, and methane (Luque et al. 2008). It is known that various stages in biofuel production encompass extremes of many physical conditions such as temperature and pH. Thus, thermophiles are the ideal entrants to replace their mesophilic analogues (Coker 2016). The other benefits using thermophilic microorganisms is that the thermophiles can easily ferment sugars from biomass or even complex carbohydrates and thus can be easily exploited in the production of second-generation biofuels (Sommer et al. 2004). Moreover, thermophilic fermentations are less disposed to other microbial contaminations. Also, product inhibition is reduced as the volatile products can be easily removed (Barnard et al. 2010).

Various thermophilic and hyperthermophilic microorganisms have been exploited in biofuel production. Although the earlier traditional methods of biofuel production such as bio-butanol and bioethanol incorporate the use of chemical processes complemented with mesophilic microbes such as *Saccharomyces cerevisiae* and *Clostridium* species (Lee et al. 2008). Other thermophiles such as thermophilic *Clostridia* (fermentative anaerobes) can degrade lignin-containing substances. They possess a multienzyme complex called cellulosome in their cell membranes. This cellulosome complex has the potential to ensure the enzymatic degradation of cellulosic substances (Demain et al. 2005). *Geobacillus* are the other promising thermophilic candidates for ethanol production. Certain species of *Geobacillus* can degrade complex carbohydrates such as xylan due to the production of xylanase enzymes by them (Wu et al. 2006). *Geobacillus stearothermophilus* produces ethanol at higher temperatures and with a good yield similar to that of *S. cerevisiae* (Bibi et al. 2014). Other species of *Geobacillus* can tolerate 10% ethanol concentration like *Geobacillus thermoglucosidasius* (Fong et al. 2006). Thus, thermophilic microorganisms have produced a great amount of interest in biofuel production.

13.4.1.3 Bioremediation and Biomining

Substantial metal contamination speaks to an essential issue because of its dangerous impact and aggregation all through the natural pecking order which prompts genuine environmental and medical issues (Najar 2018). From environmental perspectives, the removal and recovery of heavy metals are very important (Nourbakhsh et al. 2002). Several reports on eubacteria and fungi are available which deals with metal tolerance (Cánovas et al. 2003). However, hyperthermophilic bacteria were also established for bioremediation of heavy metals at higher temperatures (Rajendran

et al. 2003; Sar et al. 2013). It has also been known that there is active (bioaccumulation) and passive (adsorption) uptake of heavy metals by microorganisms (Hussein et al. 2004). A thermophilic bacterium *Anoxybacillus flavithermus* possess metal binding capacity showing less affinity toward Cu than Mn was enhanced through forming metallo-ligand complexes with -COOH, -PO_3, and –OH moieties (Burnett et al. 2007). Also, various thermophilic genera such as *Bacillus, Anoxybacillus, Brevibacillus*, and *Geobacillus* were investigated for sensitivity and adsorption of Cd (Hetzer et al. 2006). Temperature, pH, inoculum metal concentration, contact time, and biomass concentration were found to be the main conditions for equilibrium adsorption of Zn, Mn, Cd, Ni, and Cu for *G. thermoleovorans* sub species *stromboliensis* and *G. toebii* sub species *decanicus* (Özdemir et al. 2009).

Biomining generally called bioleaching, is the amputation of insoluble metal sulfides or oxides by using microorganisms (Donati et al. 2016). It has been estimated that the extraction rates using biomining are around 90% compared with 60% for traditional heap leaching (Vera et al. 2013). Biomining has been potentially exploited in the mining of various metals such as gold, copper, silver, nickel, zinc, and uranium (Donati et al. 2016). The use of thermophiles has various advantages for biomining as compared to their mesophilic counterparts. The exploitation of thermophiles reduces the possibilities of acid mine drainage (AMD) which is sometimes caused by mesophiles is the acidic water, created by the oxidation of sulfides from the mine, starts streaming or filtering out of the mine and is cost-effective (Sheoran et al. 2010). Many thermophilic strains, such as *Sulfolobus* and *Metallosphaera* have also been employed in biomining (Vera et al. 2013). Thus, the above studies suggest that the thermophilic bacteria and archaea are suitable candidates that can be potentially exploited in bioremediation and biomining (Deming and Baross 2001).

13.4.1.4 Starch Hydrolyzing Enzymes

Starch is one of the most important carbohydrate polymer made of two fractions amylopectin and amylose (Drauz and Waldmann 2012). Amylopectin molecule is a highly branched polysaccharide composed of D-Glucose residues linked with alpha 1–4 linkages and the branches are linked by α (1–6) linkages whereas the amylose molecule is a linear polysaccharide composed of D-Glucose residues linked with alpha 1–4 linkages (Robyt 2008). The degree of polymerization and the comparative content of the monomers depend on starch sources (Drauz and Waldmann 2012). Depolymerization or degradation of starch is carried out by enzymes known as amylases. Amylases are synthesized by animals, plants, and microorganisms and classified as alpha (α), beta (β) and gamma (γ) amylases. Alpha (α) amylases are endo-acting enzymes leading to the hydrolysis of α (1–4) linkages randomly and are unable to break α (1–6) linkages, thus α-amylases can lead to the formation of linear, branched oligosaccharides and limit dextrins. Beta (β) amylases are exo-acting and leading to the hydrolysis of only α (1–4) linkages. These acts on the polysaccharide chain from their nonreducing end, resulting in the formation of major oligosaccharide maltose. Gamma (γ) amylases are exo-acting and attack the substrate from

nonreducing ends which leads to hydrolysis of both α (1–4) and α (1–6) linkages thus results in the formation of monosaccharides as a major product (Abd-Elhalem et al. 2015; Horváthová et al. 2001; Sen et al. 2014). The amylases can be exploited through wide options or applications like fermentation, textile, food, detergent, pharmaceutical, etc. (de Souza and de Oliveira 2010). Since the hydrolysis of starch related to industrial progressions requires high temperature and pH. Thus, thermophilic amylases would be the first choice for industrial purposes and thus it is not surprising that thermophilic amylases have several applications in industrial microbiology (Coker 2016). Many thermophilic microbes have shown significant amylolytic activity such as *Sulfolobus acidocaldarius*, *Sulfolobus solfataricus*, *Thermophilum, Desulfurococcus, Thermococcus,* and *Thermotoga*. It was shown that *Thermotoga maritima* a thermophilic bacterium possesses all the three amylolytic properties i.e., α, β, and γ amylase activity. Also, *Pyrococcus furiosus* and *Pyrococcus woesei,* hyperthermophilic bacteria were reported to possess highly thermostable amylolytic activities (Najar 2018).

13.4.1.5 Proteases

Proteases are the class of enzyme that converts the protein into amino acid and peptides. They are classified according to the nature of their catalytic activity (Ellaiah et al. 2002). Today the quantity of commercialized protease production in the world is as large as compared to the other biotechnologically modified enzymes. In leather, pharmaceutical, food, and textile industry, these are the major used enzymes (Li et al. 2012). Serine alkaline protease is used in addition to detergents for laundering. The proteases that can catalyze responses under outrageous condition i.e., high temperature and extraordinary pH are profitable for modern applications (Drauz and Waldmann 2012). Extremophilic proteases usually are serine rich structure which renders them thermostable at high temperature despite in presence of detergents (Ellaiah et al. 2002). Many studies have been done on thermophilic bacteria and archaea to get the promising proteolytic enzymes. A hyperthermophilic archaeon *Thermococcus kodakarensis* KOD1 was studied and a highly heat-stable protease enzyme Tk-subtilisin has been isolated. It has also been shown that after recombination, this enzyme shows optimal activity at 100 °C and was readily stable under high concentrations of various denaturants (Koga et al. 2014). Another thermopsin like protease SsMTP-1, thermostable, and pH was isolated from *Sulfolobus solfataricus,* a thermophilic archaeon (Gogliettino et al. 2014). The thermophilic bacteria have also been exploited such as a thermophilic bacterium *Coprothermobacter proteolyticus* (Toplak et al. 2013). From this bacterium proteolysin (serine protease) was isolated and is an excellent candidate in the detergent industry due to its extreme stability at high temperature and elevated pH ranges.

13.4.1.6 Lipases

Lipases hydrolyses the amalgamation of esters from glycerol and long-chain unsaturated fats. They are viewed as the most flexible proteins of the enterprises which achieve a scope of bioconversion response (Sharma et al. 2011), which incorporates

hydrolysis, inter esterification, esterification, and acidolysis (Andualema and Gessesse 2012). The esters produced by lipase catalysis are crucial in the food and beverage industry for enhancing the taste and aroma (Najar 2018). Other products formed from lipase activity such as long-chain CH_3- and C_2H_4- esters of COO- moieties are used as diesel engine fuels (Jeong and Park 2008). On the other hand, ester of long-chain COO- and -OH moieties are used as additives or lubricants in cosmetics (Andualema and Gessesse 2012). The lipases have been also exploited in various other applications such as in the paper industry, casein hydrolysis in the dairy industry, non-cellulosic impurities removal from pre-processed raw cotton, drug formulations in pharmaceuticals industries, and subcutaneous fat removal in the leather industries (Andualema and Gessesse 2012). Lipases extensively produced by various microbial communities like bacteria, fungi, and yeast (Sharma et al. 2011). There are many reports on the *Bacillus* sp., as the major contributor and producer of lipolytic enzymes. In order to tolerate the extreme conditions of temperature or pH, the hunt for thermophilic lipolytic enzymes was carried out. The isolation of two thermostable and alkaline lipolytic enzymes was purified from two thermostable archaea *Sulfolobus acidophilus and Pyrobaculum* sp. (Shao et al. 2014; Zhang et al. 2014). Other thermophilic species were also being exploited for lipolytic enzymes such as *Thermotoga maritima* and *Thermus thermophilus* (Wei et al. 2013). It has also been shown that the thermophilic *bacillus* produces lipase enzymes with greater activities and stabilities than their mesophilic analogs. Thus, these stabilities of thermophilic bacteria and their enzymes signify exceptional entrants for industrial applications.

13.4.1.7 Other Enzymes

There are various other enzymes studied from thermophilic bacteria and archaea such as cellulases, esterases, pullulanases, dehydrogenases, pectinases, chitinases, isomerases, xylanases and DNA-modifying enzymes. Cellulose is the most abundant polymer on earth can be hydrolyzed by complex enzymes known as cellulases. Cellulase enzymes include endoglucanase, exoglucanase, and β-glucosidase (Acharya and Chaudhary 2012). Cellulases are important catalysts in various industrial applications such as food, detergent, textile, pulp, and paper. These cellulases can also be exploited in ethanol production (Kuhad et al. 2011). Various thermophilic bacteria have been studied such as an anaerobic thermophile *Clostridium thermocellum* has shown cellulolytic activities. Thermophilic *Bacillus* isolated from hot springs (India) also has shown cellulolytic activity (Acharya and Chaudhary 2012). Other highly thermostable cellulases stable at temperatures between 95 and 115 °C has been isolated from *Thermotoga maritima* MSB8 and *Thermotoga* sp. FjSS3-B1 (Najar 2018). Similarly, other enzyme such as esterase has been studied in some thermophilic microorganisms. Esterases catalyze the hydrolysis of ester bonds (Bornscheuer 2002). Thermo-stable acetyl xylan esterases are of great interest nowadays and have been also extracted from anaerobic microbes *Clostridium thermocellum*, *Thermoanaerobacterium* sp., etc. The phenolic acids are the precursors to many by products and can be explored in biorefineries and can also be exploited in food and cancer gene therapy (Sood et al. 2016). Besides these

above-discussed applications of thermophilic microorganism and their thermo stable enzymes, there are many other fields where they can be significantly exploited in the fields of agriculture, dairy, medical, cancer treatments, etc.

13.4.2 Psychrophiles and Cryozymes

With the advancement of science and technology, the world has reached a different stage today. Working at the molecular level has led to the age of genomics and systems biology that have made possible the processes that find applications in agricultural, food, medical, and textiles industries. Moreover, due to increasing environmental concerns, more emphasis is being given upon natural, biological ways of processing and production rather than not so eco-friendly chemical means. In this aspect, enzymes are finding good applications in industries; particularly cold-adapted enzymes are of great potential for biotechnological application (Margesin et al. 2007; Miteva 2008). Much of the earth's biosphere is permanently cold (Priscu and Christner 2004). Organisms that thrive in such harsh climate, comprising of members from archaea, bacteria and eukarya, are successfully adapted to their environment (Cavicchioli 2006; Deming 2009; Margesin et al. 2007). Their adaptations enable them to grow and perform metabolic activities similar to their mesophilic and thermophilic counterparts. Hence, psychrophiles and psychrotrophs serve as natural reservoirs for enzymes that can function actively at low temperatures, and these cold-active enzymes have huge biotechnological potential (Cavicchioli et al. 2011). These cold-evolved enzymes with high catalytic efficiency are referred to as cold-active enzymes (Cavicchioli et al. 2011; Ramana et al. 2000). These are temperature sensitive on comparison with mesophiles and thermophiles. All cryozymes share one common property: thermo labile activity. The active site of the enzyme is assumed to be most heat-labile in nature. The low stability and heat-labile activity are the results of increased flexibility of the protein or its active site (Feller 2017). High flexibility to the thermo labile cold-adapted enzymes is contributed by many factors such as decreased H-bonding and other electrostatic interactions, lesser core hydrophobicity, enhanced surface hydrophobicity, longer loops with lesser proline residues, increased glycine residues, lesser disulfide bridges, etc. (Cavicchioli et al. 2011). They include protease, alpha-amylase, lipase, cellulase, esterase, xylanase, DNA ligase, alkaline phosphatase, chitinase, pectinase, alpha-lactamase, and many more (Joshi and Satyanarayana 2013). Cold-active enzymes have found huge industrial and biotechnological applications owing to high specificity at low temperature, highly thermolabile nature at increased temperature (Joshi and Satyanarayana 2013). Cryoenzymes are economical to use thereby saving energy costs. Due to their heat-labile nature, these cold-active enzymes are very useful in case of enzyme reactions where heat-sensitive substrates are being handled and it avoids undesirable by-products (Cavicchioli et al. 2011). Because of these properties, cryo-enzymes are the most useful in the food and beverage industry where maintenance of nutritional and functional value of food, their flavor and taste is very important (Joshi and Satyanarayana 2013). Cryo-proteases comprise of a

significant group of enzymes as they are hydrolytic enzymes involved in most of the physiological and metabolic functions. It has been seen that at present, more than 70% of commercialized enzyme is proteases. Cryo-proteases are widely used in various fields such as food and dairy, baking, pharmaceuticals, cosmetics, textiles, leather processing, environmental bioremediation, and many more (Joshi and Satyanarayana 2013). Psychrophilic proteases have been modified to enhance their stability and catalytic activity. They seem promising for future aspects as efficient therapeutic agents (Ramana et al. 2000). Along with their wide industrial applications, cold-active enzymes are equally important in molecular sciences. Alkaline phosphatase is one of the important DNA-modifying enzymes extracted from psychrophiles. Another novel cold-adapted cellulase enzyme has also been discovered that seems very useful as at low temperatures it can convert cellulosic waste materials to biofuel (Cavicchioli et al. 2011). A wide range of psychrophiles as well as psychrotrophs produce various cold-active enzymes that can optimize the cost and efficiency of present-day industrial processes. There is scope for the development of newer techniques with lesser energy input and thereby reduction in cost by removal of cost for heat inactivation step (Joshi and Satyanarayana 2013).

13.4.2.1 Biotechnological Importance of Psychrophiles

Cryozymes have created particular interest in food and beverage industries because of their functioning at low temperatures, which will minimize the incidence of spoilage and alteration of nutritional value, taste, or quality of the product. Cold-active proteases can enhance the organoleptic property of frozen meat products (Margesin et al. 2007). Cold-active proteases, lipases, amylases, and xylanases are useful for baking in preparation of dough and processing, giving larger volumes of dough (Joshi and Satyanarayana 2013). Cryozymes have importance in pharmaceutical industries. Increasing concern for pure drugs has led to the need of biocatalysts for organic synthesis (Margesin et al. 2007). A heat-labile lipase from *Candida antarctica* has been isolated and applied broadly in modification of polysaccharide, resolution of alcohols, etc. (Margesin et al. 2007). Cryozymes have wide uses in cosmetic industries as they enhance biotransformation reactions, thus preserving the volatile substrates like fragrance (Margesin et al. 2007).

13.4.2.2 Application in Molecular Biology

Even in molecular biology, cold-active enzymes play vital reactions in sequential reactions. Alkaline phosphatase dephosphorylates vector priming prior to cloning so that self-ligation does not occur, and for removing 5’phosphate group from DNA before end-labeling (Cavicchioli et al. 2011; Margesin et al. 2007). The heat-labile nature of cryozymes is beneficial as it can be heat-inactivated after its function is over. Cold-adapted ligases can provide a better yield of ligation at low temperatures and thus is advantageous over mesophilic ligases (Margesin et al. 2007). Cold-active enzymes are further being studied for their exploitation in various biotechnological and molecular fields, to provide a convenient, economical, and cost-effective means.

13.5 Diversity and Enzymology of Thermophilic Bacterial Isolates from Hot Springs of Sikkim

The culture-dependent isolation of the thermophilic bacteria producing industrially important enzymes was done from four different hot springs of Sikkim. The culture-dependent studies showed the complete dominance of phylum Firmicutes in the hot springs of Sikkim. *Geobacillus* was the predominant genus along with few representatives of *Anoxybacillus* and *Bacillus*. Our study through culture study showed that—*G. stearothermophilus* XTR25, *G. kaustophilus* YTPR1, *G. subterraneus* 17R4, *G. lituanicus* TP11, *G. kaustophilus* YTPB1, *Parageobacillus toebii* 10PHP2, *G. toebii* strains, *Anoxybacillus caldiproteolyticus* TRB1, *Anoxybacillus gonensis* TP9, *Bacillus smithii* 17R6, *Bacillus* sp., 17R5 were the bacterial flora present respectively. A novel bacterium was also isolated from Yumthang hot spring—*Geobacillus yumthangensis* (Najar et al. 2018a, b, c).

Culture-independent analysis through metagenomics of the hot springs of Sikkim showed various phylum diversity like—Proteobacteria (~63%), Bacteroidetes (~15%), Acidobacteria (~4%), Nitrospirae (~4%) and Firmicutes (~3%) in Borong *Tatopani*; Polok *Tatopani* had Proteobacteria (~47%), Bacteroidetes (~4%), Firmicutes (~3%), Parcubacteria (~3%) and Spirochaetes (~3%); Yumthang *Tatopani* had Actinobacteria (~98%) and Proteobacteria (~2%) in majority; Reshi *Tatopani* had Proteobacteria (~76%), Actinobacteria (~23%), Firmicutes (~1%), and Cyanobacteria (0.03%).

At genus level there was a distinct variation in hot springs. The genera present in Borong *Tatopani* had *Acinetobacter* (~8%), *Flavobacterium* (~4%), *Vogesella* (~4%), *Ignavibacterium* (~3%), *Sediminibacterium* (~3%), *Thermodesulfovibrio* (~3%), and *Acidovorax* (~2%); Polok *Tatopani* had *Flavobacterium* (~3%), *Sediminibacterium* (~3%), *Pseudomonas* (~2%), *Treponema* (~2%) and *Opitutus* (~1%); Yumthang *Tatopani* had *Rhodococcus* (~98%), *E. coli* (~0.7%), *Serratia* (~0.5%), *Nocardiopsis* (~0.5%), *Brevundimons* (~0.2%) and *Acinetobacter* (~0.2%); Reshi *Tatopani* had *Pseudomonas* (~85%), *Rhodococcus* (~4%), *Dietzia* (~4%), *Arthrobacter* (~4%), *Staphylococcus* (~1%), and *Paracoccus* (~0.3%).

The diversity at species level varied significantly in all the four hot springs. Polok *Tatopani* had *Sediminibacterium goheungense, Opitutus terrae*, *Treponema caldarium, Ignavibacterium album, Desulfobulbus mediterraneus, Thermodesulfovibrio yellowstoni*, *Hydrogenobacter thermophilus, Thermoanaerobacter uzonensis, Thermoanaerobaculum aquaticum, Thermodesulfovibrio hydrogeniphilus, Thermolithobacter ferrireducens, Thermus arciformis, Thermus caliditerrae,* etc. Borong *Tatopani* had *Ignavibacterium album*, *Rheinheimera aquatic*, *Flavobacterium cheonhonense*, *Thermodesulfovibrio yellowstoni*, *Thiovirga sulfuroxydans*, *Meiothermus hypogaeus,* etc. Reshi *Tatopani* had *Microbacterium species* (~67%), *Arthrobacter phenanthrenivorans* (~3%), and *Rhodococcus erythropolis* (~2%) and Yumthang *Tatopani* had *Rhodococcus ruber* (~98%) and *Escherichia coli* (~1%), respectively. Polok and Borong *Tatopani* had lesser amount of archaeal communities. Borong *Tatopani* had *Crenarchaeota* (~1%), whereas Polok *Tatopani* had *Euryarchaeota* (~0.6%). *Desulfurococcales*

and *Desulfurococcus* were the major order and genus under *Crenarchaeota* respectively whereas *Methanomicrobiales* and *Methanospirillum* were the major order and genus under *Euryarchaeota*. However, we could not find any archaeal communities in Reshi and Yumthang *Tatopani*.

Our preliminary research on α-amylase enzymatic production showed that there were few isolates that had this property. It was found that the enzyme was functional at 60 °C and AYS8 isolate showed the highest enzymatic activity of 2.6 Units min^{-1} mL^{-1} while SY10 showed the lowest activity of 2.1 Units min^{-1} mL^{-1} at 60 °C. However, enzyme production was highest at 37 °C. The isolate AYS 8 showed the highest enzyme activity of 2.76 Units min^{-1} mL^{-1} at pH 8. α-amylase enzyme produced by the isolates isolated from Yume Samdung and Yumthang hot springs of Sikkim, indicated the enzyme was thermostable from 37 °C–90 °C (optimum = 60 °C), but isolates showed two different optimum activity at pH 7 and 8. Thermo stability is one of the important characteristics for industrial applications.

13.6 Diversity and Enzymology of Psychrophilic Bacterial Isolates from Glaciers of Sikkim

Our study in the Changme Khangpu (CK) and Changme Khang (CKG) glaciers of Sikkim through culture-dependent studies showed that the bacteria population was dominated by phylum Firmicutes and belonging mostly to *Bacillus* genus (*Bacillus cereus* KY982961; *Bacillus thuringiensis* KY982962; *Bacillus safensis* MF163138, *Bacillus oceanisediminis* MF163139, *Bacillus nealsonii* MF163141, and *Brevibacillus brevis* MF191718). The second most dominant phylum was Actinobacteria and identified belonged to *Neomicrococcus lactis* MF163142, *Pseudoclavibacter terrae* MF163143, and *Brevibacterium linens* MF1631143. The bacteria identified from Kanchengayao glacier belonged to *Pseudomonas* genus with different species such as *P. fluorescens, P. reactants, P. hibiscicola, P. maltophilia, P. synxantha, P. poae, and P. azotoformans.* The identified bacteria from Chumbu glacier belonged to *Bacillus* groups such as *Bacillus wiedmannii*, *B. velezensis, B. odorifer, and B. fusiformis* (Sherpa et al. 2018).

Culture-independent studies through metagenomics of CK glacier revealed that at phylum level, Proteobacteria were (~99%) abundant followed by unidentified virus (~0.2%), Firmicutes (0.03%), Ascomycota (0.001%), and Actinobacteria (0.14%). At class level, CK had *Beta-proteobacteria* (~66%), *Gamma-proteobacteria* (~18%), *Alpha-proteobacteria* (~15%), *Bacilli* (~0.4%), *Actinobacteria* (~0.3%), *Eurotiomycetes* (0.009%), and unidentified virus (0.27%). At the genus level, CK glacier had *Delftia* (~62%), S*erratia* (~17%), *Brevundimonas* (~15%), *Massilia* (~3%), and *Bifidobacterium* (~0.02%). Species-level classification showed the dominance of *Delftia acidovorans* (~38%), *Delftia* unclassified (~25%), *Serratia marcescens* (~17%), *Brevundimonas* unclassified (~15%), and *Massilia* unclassified (~3%).

Glacier CKG metagenomics at phylum level had Proteobacteria (~99%), followed by unidentified virus (~0.2%), Firmicutes (~0.03%), Ascomycota (~0.001%) and Actinobacteria (~0.1%). At class level classification, CKG had *Beta-proteobacteria* (~52%), *Gamma-proteobacteria* (~36%), *Alpha-proteobacteria* (~11%), *Bacilli* (~0.04%), *Actinobacteria* (~0.1%), *Eurotiomycetes* (~0.01%), and unidentified virus (~0.24%). At genus level classification, CKG glacier had *Delftia* (~49%), *Serratia* (~31%), *Brevundimonas* (~11%), *Stenotrophomonas* (~3%), *Massilia* (~2%), *Commamonas* (~0.7%). Species-level classification were dominated by *Serratia marcescens* (31%) followed by *Delftia acidovorans* (~29%), *Delftia* unclassified (~20%), *Brevundimonas* unclassified (~11%) and *Stenotrophomonas maltophilia* (~3%). Highest amylase activity was showed by isolated CK13 *Bacillus safensis* (1.07 Units mL^{-1} min^{-1}) on the other hand highest protease activity were showed by isolated CKG2 *Bacillus thuringiensis* (2.24 Units mL^{-1} min^{-1}) (Sherpa et al. 2018).

13.7 Biotechnological Potential of Polyextremophilic Bacterial Isolates

Tolerating or adapting in extreme conditions varying multi parameters like pH, salinity, and temperature is a very unique biological adaptability and these special microbes are called polyextremophiles (Chela-Flores 2013; Dhakar and Pandey 2016). They might be optimally functioning in varying the acidity or alkalinity accompanying high salinity with hyper-temperate conditions (temperature > 70 °C or methane gas conditions). The polyextremophiles have recently gained lots of research momentum. Research perspectives to understand the "know-how" mechanisms for surviving the pertinent niche sturdiness and their possible biotechnological and industrial applications.

During our research, we also have encountered such resilient species from the hot springs which showed optimal growth at wide ranges of pH and temperature. Our isolates although did not prefer high salinity rather liked minimal saline conditions present without adding any NaCl in the media. There have been many reports in extremophilic bacteria isolated from harsh environments that had neutral pH "on-field" but microbiological characteristics showed that they were able to withstand a wide range of pH (1–12) during in vitro studies. There have been reports of many such bacterial isolates like *Bacillus* and *Paenibacillus* sp. from few hot springs in India which can endure varying temperature ranges (20 °C–80 °C) and wide range of pH (5–14) (Pandey et al. 2014a). *Geobacillus stearothermophilus* (GBPI-16), obtained from Soldhar hot spring, India not only survived the autoclave conditions but can grow actively at 95 °C and produce very stable amylase and lipase (Pandey et al. 2014b). Initial studies with these isolates are encouraging, however, it required more research in these kinds of polyextremophilic bacteria. The survival mechanisms might lie with the genomic structural organization and extremozymes which renders them such poly-phasic extreme properties. The enzymes from these

polyextremophilic bacteria might also have poly-phasic extreme properties which may be have more biotechnological applications.

Psychrotolerant species are other exceptions which at the lowest freezing conditions again can survive such harsh conditions and produce metabolic by-products at a wide range of temperatures and pH (Dhakar and Pandey 2016). These special characteristics feature can help the food and beverage and pharmaceutical industries. In the natural habitat, these tolerating features also in the future can relate to the ecological succession when glacier retreats occur and the soil gets exposed. They also participate in various biogeochemical pathways to recycle and replenish the nutrient cycles for e.g., the carbon cycle, nitrogen cycle, and phosphorous cycle at sub-zero temperatures (Dhakar and Pandey 2016). There have been many reports of such microbes isolated from different Himalayan regions of India and their role in various ecological phenomena like biomining or bioremediation, biocontrol, or plant growth-promoting lie characteristics. A classic example is that of the most predominant micro-flora, the psychrotolerant strain of *Serratia marcescens* (Dhakar and Pandey 2016; Sherpa 2018). Even during our study, we found this bacterium and its cohabitants were *Bacillus* and *Pseudomonas*—all famous for their quorum sensing approaches.

13.8 Conclusions

The State of Sikkim is one of those hot spots in the Himalayas where both hot and cold microbial ecology exists. And this very subtle coexistence of natural habitats enables us the freedom as researchers to explore the fundamentals of life. Ecology is very sensitive in the Himalayas as they are subjected to climatic conditions.

Our study reported here focused only on a few hot springs and glaciers. Bioengineering of industrially beneficial strains is one of our prime interest and holds a significant stake. From the hot springs, the average temperature of all our sampling sites varied from 40° to 70 °C depending on the seasons. They naturally harbor many polyextremophiles which can grow in a wide range of temperatures and pH. We have also found many novel species whose genetic makeup is unique with less (G + C) content and interesting morphology. Most of our bacterial isolates are *Geobacillus* and these are pretty exciting microbes to explore in the near future. As some of the isolates are good amylase and protease producers, this very potential has to be engineered further to step up the enzyme production and clone the genes. Thus, exploration has just begun and many more steps are yet to be taken.

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Enzymes in Health Care: Cost-Effective Production and Applications of Therapeutic Enzymes in Health Care Sector

14

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Abstract

Application of enzymes in health care sector is becoming a popular therapeutic area of treatment. Recent advances in modern biotechnology have revolutionized the development of new enzymes. It is not surprising that the enzymes derived from wild as well as genetically engineered microbes coupled with protein engineering have gained interest for their widespread uses in pharmaceutical industries and medicine owing to their higher stability, catalytic activity, ease of production and optimization than that of plant or animal derived enzymes. In recent times, the scientific world has perceived the importance of bacterial protease in matrix destruction and modulating host response, thereby exhibiting significant importance in curing odontal disease. Moreover, bacterial collagenase has been prominently used to treat skin diseases and inflammatory periodontal disease. Another microbial enzyme, amylase has also been found to be applicable in emerging field of health care like enzyme replacement therapy. Microbial chitinase can be used in the treatment of several infections. Microbial lipases find applications as digestive aids and in the treatment of necrosis. This book chapter will revolve around aspects like the production of microbial enzymes and

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their inevitable role in health care sectors like wound healing, assisting metabolism, drug delivery and related diseases.

Keywords

Microbial therapeutic enzymes · Enzyme therapy · Fibrinolytic enzyme · Lipase · Collagenase · Chitinase · Uricase

14.1 Introduction

The interest of investigators in exploring enzyme technology, a burgeoning segment of industrial biotechnology, is gaining impetus with ongoing time. Enzyme technology involves research on fundamental and applied enzymology, predominantly in biocatalysis, diagnostics, therapeutics, structural and molecular modelling. The comprehensive aim of this technology is concerned with development of new and more sustainable enzymes or enhancing production of prevailing enzymes from new raw materials and biomass, following an inexpensive, efficient and eco-friendly direction. Thus, the overall objective makes enzyme technology a promising and popular space in pharmaceutical research and health care sector of today's world, where diagnosis, therapy, biochemical investigations and monitoring of various alarming diseases can be performed conveniently, effectively and economically.

Significant scientific works on use of enzyme in resisting infections have unlocked the utility of enzymes in all dimensions. One of such significant work was carried out by researchers who have found that extra cellular secretions of *Bacillus pyocyaneus* kills anthrax bacilli and protects the infected mice (Mane and Tale 2015). Subsequently, similar scientific works had paved the path for using enzymes as therapeutics. Researchers had noticed that crude proteolytic enzymes could be used in treating gastrointestinal disorders. Later, combination of enzymes had been employed in combating infections where bacterial phage associated lysing enzymes like lysin and lysostaphin were used for treating dental carries (Fischetti et al. 2002). Scientists had even conducted studies where combination of enzymes along with antibiotics had successfully served in treating multi organ failure and sepsis (Ahmed et al. 2014).

While talking about enzymes, history has demonstrated the active participation of microbes in production of commercially important enzymes (Adrio and Demain 2014). With time, there have been constant updates on isolation and physicochemical characterization of microbial enzymes. Since these microbial enzymes hold huge industrial importance, studies related to their production from lab scale to pilot scale and their operations in biomedical industries have continuously progressed (Nigam 2013). The crucial role of microbial biotechnology in producing medically valid biomolecules like antibiotics, hormones, alkaloids, vitamins and enzymes had been also explored (Sikyta et al. 1986). Generally, medically crucial enzymes are derived from restricted number of specific fungi and bacteria (Mane and Tale 2015). Microbes have been used as elementary source of enzymes production as these

microbes can be cultured in enormous numbers in restricted time period. On top of all, bacterial cells can be subjected to genetic manipulation to enhance the production of enzyme. The microbial enzymes have witnessed more popularity because of their consistency in yield, activity and productivity than that of the enzymes derived from plants and animals (Gurung et al. 2013). As majority of these microbial enzymes exhibit low K_m (Michaelis constant) and high V_{max} (Maximum velocity), so these enzymes display maximum efficiency at low concentration of enzyme and substrate (Mane and Tale 2015). Above all, the microbial enzymes are more active, stable and do not exhibit seasonal fluctuations in productivity. Thus, all these assets of microbial enzymes played a pivotal role in attracting the attentions of researchers in microbial enzyme technology (Anbu et al. 2017). With continuous research, the problem associated with the production of enzymes under extreme conditions has also been addressed. Culturing them under intense conditions have made these microorganisms adaptable for bio-synthesizing, especially medically critical enzymes (Nigam 2013).

Recent advancement in modern biotechnology has refashioned the development of new enzymes. It is not surprising that the microbial enzymes derived from wild as well as genetically engineered microbes coupled with protein engineering have been in rampant uses in pharmaceutical industries as these enzymes can be tailor made, blend and used in mimicking the natural and physiological functioning within the living cells of the human body. One of the prime issues of using microbial enzymes as therapeutics has been the purification process. These enzymes should be purified from all unwanted, incompatible and toxic contaminants. So, voluminous studies have been conducted for analyzing the meticulous details in developing fermentation and purification processes of the concerned enzymes. These developments have eventually paved the path for adopting these purified and well characterized enzymes in pharmaceutical industries on pilot scale. In recent times, employment of immobilized cells technique for production of enzymes is gaining more acceptance in comparison to the free cell system, as researchers have experienced higher rate in production and easy separation of products in case of immobilized cells technique (Mateo et al. 2007). Currently, investigations have been conducted on the application of genetic engineering techniques where microbial genome has been manipulated in an effort to improve the production, activity and adaptability of the enzymes. The properties of the enzymes like binding sites, catalytic sites, etc. can be even altered by the looming technology of protein engineering (Huston et al. 1988). Furthermore, desired properties can be incorporated in the protein of interest by employing site directed mutagenesis. Thus, recombinant DNA technology coupled with protein engineering has revolutionized the commercialization of medically imperative enzymes.

14.2 Enzyme Therapy

Enzyme therapy refers the use of natural and engineered enzymes for therapeutic purpose in health care sector. It has arrested the distinctive attention of scientists all across the world because of its alluring applications in treatment of cystic fibrosis,

pancreatitis and even against tumour cells. This therapy can treat enzyme deficient disease by administering engineered enzymes into the body. Enzyme therapy can be systemic or non-systemic. The concerned therapeutic enzymes can be introduced into the human body via multiple routes like orally taken pills and capsules, topically administered ointments or intravenously taken injections. Systematic enzyme therapy refers to a process that uses mixtures of enzymes for assisting the various regulatory and communication systems of the body. Clinical studies have been conducted where proteolytic enzymes concoction is prepared with combination of papain, trypsin and chymotrypsin for killing cancerous cells. The advantage of this systemic enzyme therapy in cancer treatment lies in curtailing the injurious effects caused by the conventional radiotherapy and chemotherapy (Leipner and Saller 2000).

One of the protagonist research studies that had been done in this context is the pancreatic enzyme therapy, where the gastrointestinal physiology is restored in the patient's body by supplementing the deficient pancreatic enzymes orally (Domínguez-Muñoz 2007). Pharmaceutical preparation comprising of concoction of microbial enzymes namely, lipase, protease, amylase obtained from fungal strains of *Rhizopus delemar*, *Aspergillus melleus* and *Aspergillus oryzae* respectively has gained colossal success in treating maldigestion in humans and other mammals (Galle et al. 2004). Scientific work also states that the chronic pain associated with pancreatitis may get reduced by the help of this therapy (Domínguez-Muñoz 2007).

Chymotrypsin is studied to have the potential in combating inflammation and infection. It is taken orally as drug against gastric ulcers and also known to reduce liver stress. This enzyme is even a potent medication against pulmonary diseases like asthma. *Escherichia coli* can be genetically modified by introducing the cDNA which encodes for chymotrypsinogen from *Metarhizium anisopliae* (Volontea et al. 2011). Chymotrypsin, possessing anti-inflammatory, disinfectant, antioxidant and proteolytic properties makes it a promising drug in tissue repairing (Ghaffarinia et al. 2014; Shah and Mital 2018).

Deficiency of enzyme glucocerebrosidase leads to a rare genetic disease termed as Gaucher disease or lipid storage disorder. The pathophysiology of the disease points out the aggregated glucocerebroside within the cells of macrophage-monocyte system resulting in symptoms like swelling of lymph nodes, liver malfunction, enlarged spleen, bone lesions and skeletal disorders. Enzyme replacement therapy for treating Gaucher disease with recombinant glucocerebrosidase enzyme produced from Chinese hamster ovary has been a notable success in medical technology (Pastores 2010). Enzyme replacement therapy for treatment of lysosomal storage disorders acquired its impulse from the achievement of the treatment of Gaucher disease. Recent research on enzyme therapy revealed that the proteolytic enzymes of phage virus are very potent therapeutics against multidrug resistant bacterial disease (Lin et al. 2017). Thus, it is worth mentioning that ongoing worldwide research on enzymes and the technologies associated to make novel use of these enzymes in treatment of diseases has brightened up a new dimension in healthcare sector.

14.3 Enzyme Engineering

With the rolling advancement on using enzymes in medical treatment, it has become mandatory to give voice to the engineering approaches that have been successful in enhancing the enzyme in all its dimensions, starting from activity, stability, production, purification to designing novel enzyme and incorporating desired properties to the enzyme. An array of diverse enzyme engineering methods like directed evolution, media engineering, active site imprinting, process engineering, de novo protein design with help of bioinformatic tools, etc. are feasible in recent times (Allewell 2012). The comprehensive objectives of enzymology research are concerned with the exploitation of novel enzymes, improving the catalytic and binding properties of the existing enzymes, production system through appropriate bioreactor designing and downstream processing for enhanced yield and recovery. Subsequently, standardized methods have been implemented in achieving the objectives:

1. Novel enzymes have been screened from natural samples with improved properties.
2. The existing enzymes have been genetically engineered to procure the desired characteristics.
3. The refining of the downstream processing in enzyme production has been executed.
4. Different approaches in formulation of enzyme concoction preparation have been extensively studied to combat health disorders (Li et al. 2012).

In recent time, directed evolution is a flaming segment in protein engineering which mimic the process of natural selection to manipulate proteins for the desired purpose. In this process the concerned gene is subjected to repetitive mutations and thus creating mutagenic variants. The variant containing the desired mutation is selected, amplified and used for varied purposes (Cobb et al. 2013). Among the humongous amount of work going on in protein engineering with a major effort on bringing innovative therapeutic drugs, only two have been mentioned below.

14.3.1 Metagenomic Analysis of Microbes for Recovering Novel Enzymes

The immense success of genome sequencing technology has enabled in building up of metagenomic libraries containing whole genome sequence of microbes residing in different exotic locations from all over the world like deep sea bed, arctic tundra, volcanic vent, hot spring, etc. Mining of these libraries has provided an opportunity to explore the mammoth amount of information preserved in sequence databases. Recovery of new enzymes can be possible by digging these databases and by systematic screening of the potent novel enzyme encoding codons (Gilbert and Dupont 2011) (Fig. 14.1). Screening of metagenomic libraries for recovering the

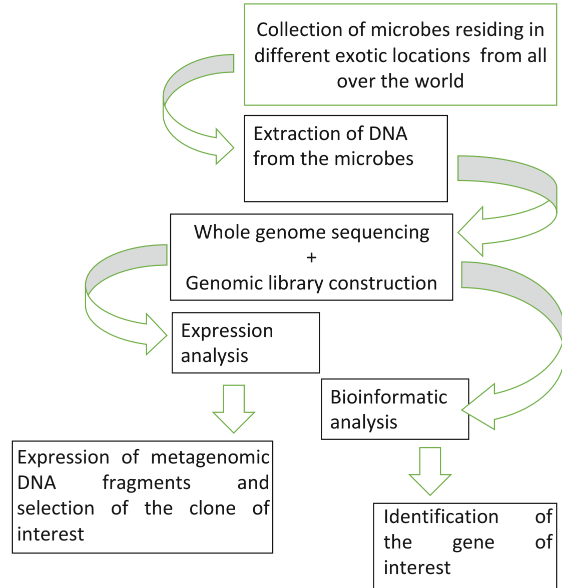

Fig. 14.1 Metagenomic analysis of microbes

desired open reading frame is predominantly based on phenotype detection, induced gene expression and heterologous complementation (Li et al. 2012).

14.3.2 Cell-Free Expression System in Protein Engineering

Cell-free expression system involves removal of native genomic DNA, transportation barriers like cell wall and cell membrane from the microbes and removal of cellular compartment. This system avoids all the restrictions of recombinant protein expression system in living cells like protein degradation (Katzen et al. 2005). One striking technique of cell-free expression system is the in vitro compartmentalization (IVC) techniques which have added a heavy momentum to the directed evolution of enzymatic activity (Griffiths and Tawfik 2003).

14.4 Need for Microbial Enzyme

The inflating global population is calling sustainable solutions to majority of the health problems. The answer to this call for viable solution lies in utilizing self-replicating and self-repairing microbiota. Microbial secretions especially in form of enzymes have continued to serve as therapeutics to the mankind. With the advent of technological advancement in health care sector, scientists have devoted monumental attention to the microbes and microbial secretions rather than chemicals. Microbial enzymes engaged in medical implication enjoyed more popularity than that of chemical pharmaceuticals owing to their high catalytic efficiency, long half-life,

consistency in yield, activity and productivity, ability to show activity in mild reaction conditions, less waste generation and pollution (Anbu et al. 2017). Fungi and bacteria have been vividly explored for production of enzymes and medically crucial enzymes are obtained from limited number of specific microbes (Mane and Tale 2015). Microbes drew acceptance from researchers because they can be grown in huge numbers within limited time interval and do not show seasonal fluctuation in producing enzymes. Above all, the microbes can be genetically modified to produce desired enzymes and to enhance the production of the needed enzymes (Demain and Vaishnav 2009). In midst of all the assets of microbial enzymes, these do suffer from certain limitations such as challenges related to their purification, requirement of cofactors and sometimes have proved to be allergens to animals. However, certain approaches can resolve these restrictions like cofactor recycling, curtailing the immunogenic property of the enzyme by help of protein engineering techniques (Yazbeck et al. 2004).

14.5 Microbial Enzymes as Therapeutics

In the field of therapeutics, enzyme therapy finds its vivid utilization in treating an array of veterinary and human disorders like blood coagulation, enzyme deficient metabolic disorders like maple syrup urine disease, genetic diseases like cystic fibrosis, phenyl ketonuria, etc. and infectious diseases caused by bacteria, fungi or protozoa (Kaur and Sekhon 2012).

Therapeutic enzymes have been of immense help in lactose intolerant people where supplements of protease, lipase and amylase have administered in these patients and have been successfully able to curtail the disease (Mane and Tale 2015). In acute lymphocytic leukaemia, aspartate ammonia ligase activity lacking tumour cells cannot synthesis L-asparagine. The asparginase create a starvation micro environment for the tumour cells. The enzyme asparginase is produced as by product by *E. coli* and can be administered intravenously in leukaemia patients (Gurung et al. 2013). Researchers cannot deny the inevitable role of cytotoxicity of RNases towards tumour cells. Extracellular ribonuclease from *Bacillus licheniformis* exhibits antitumour effect and has been captivating massive attention from cancer biologists (Anbu et al. 2017).

The cause of cardiovascular disease is often due to formation of blood clot in arteries and veins. The disease turns out to be extremely fatal if a blood clot is housed in coronary arteries. Microbial enzymes had served both as anticoagulating and thrombolytic agent for many disorders. Most extensively employed enzymes for this purpose are streptokinase from *Streptococcus* and urokinase from *Bacillus subtilis* (Adivitiya and Khasa 2017). These microbial thrombolytic enzymes had found their operations as therapeutics for ample number of thromboembolic problems in different organs of the body like deep vein thrombosis, peripheral artery disease and superficial thrombophlebitis.

Micrococcus lysodeikticus produce lysozyme which puts its attack on glycoproteins of bacterial walls and had proved to be beneficial in treating infections

of eyes, skin and gut. Lysozyme also displayed antiviral and antibacterial activity (Sizer 1972). Reports speaks that enzyme like trypsin can be efficiently used as surface disinfectants and papain can be used as deworming agents (Bielanski et al. 2013).

In therapeutics, drug delivery has been always a challenge, especially in case of brain related disease like Alzheimer's disease, where the prime difficulty lays in crossing the blood-brain barrier. The most potent drug for Alzheimer's disease is Flurbiprofen, but it cannot penetrate the blood-brain barrier. Studies have been conducted to modify Flurbiprofen with ascorbic acid which enhanced the drugs' permeability through the barrier as ascorbic acid acts as carrier for brain delivery (Anbu et al. 2017).

Table 14.1 shows a list of commonly studied microbial enzymes used in healthcare sector.

Among the entire therapeutic microbial enzyme, few enzymes have been discussed below.

14.5.1 Protease

Peptide hydrolases (EC 3.4.21–24), or, peptidases, well known as proteases bring about the hydrolysis of peptide bonds in proteins. They are widely used in detergent, pharmaceutical, tanning and food industries. The peptide hydrolases are classified under subclass 3.4 of class 3 of enzymes, i.e. the hydrolases, by the International Union of Biochemistry and Molecular Biology (Mamo and Assefa 2018; Sandhya et al. 2005).

Depending upon their catalytic site, proteases are commonly divided into the endopeptidases and exopeptidases. Endopeptidases are proteolytic enzymes that hydrolyze peptide bonds that are far from the termini of the substrate while exopeptidases hydrolyze peptide bonds near the amino or carboxy termini (Yegin and Dekker 2013). Analyzing the activity pH of proteases, they are categorized into acid, neutral and alkaline proteases (Rao et al. 1998).

Aspartic, serine, metallo and cysteine proteases comprise the four categories of proteases based on their catalytic action. The mode of action of these proteases is discussed below.

Aspartic Proteases or Aspartyl proteinases, which are mostly acidic proteases have two residues of aspartic acid within their active site, which are vital for biocatalytic ability (Yegin et al. 2011). Aspartic proteases obtained from microorganisms are mainly categorized into pepsin-like enzymes or rennin-like enzymes.

Cysteine Proteases comprise of about 20 families, produced by prokaryotes and eukaryotes (Theron and Divol 2014). This family of proteases is distributed lesser as compared to aspartic and serine proteases (Sandhya et al. 2005).

Metalloproteases are highly diversified proteases, which need divalent metal ions for being active. Of the 30 metalloprotease families, 17 include endopeptidases, while 12 include exopeptidases, and 1 comprises of both types (Rao et al. 1998).

Table 14.1 Source of therapeutic microbial enzymes

Source	Organisms	Enzymes	Applications	Reference
Fungi	*Aspergillus* sp.	Amylase	Digestion aids	Elmarzugi et al. (2014)
		Glucose oxidase	Antimicrobial	Bankar et al. (2009)
		α-Galactosidase	Prevention of xenorejection, blood group transformation	Anisha et al. (2008)
		β-Galactosidase	Removal of lactose from milk	Husain (2010)
	Aspergillus niger	Acid protease	Gastric disorder	Kaur and Sekhon (2012)
		Glucosidase	Antitumour	Kaur and Sekhon (2012)
	Aspergillus oryzae	Acid protease	Gastric disorder	Kaur and Sekhon (2012)
		Lipase	Pancreatic disorder	Kaur and Sekhon (2012)
		Maltase	Treatment against Pompe s disease	Kaur and Sekhon (2012)
	Aspergillus fumigatus	Nonribosomal peptide synthetase	Inhibit cell division by preventing microtubule formation, thus prevent tumour	Deirdre and Claire (2007)
	Aspergillus flavus	Uricase	Treatment for gout	Terkeltaub (2009)
	Beauveria bassiana	Peptidase	Celiac disease	Kaur and Sekhon (2012)
	Candida lipolytica	Lipase	Pancreatic disorder	Kaur and Sekhon (2012)
	Candida rugosa	Lipase	Pancreatic disorder	Kaur and Sekhon (2012)
	Penicillium sp.	Glucose oxidase	Antibacterial	Kaur and Sekhon (2012)
		Penicillin acylase	Production of antibiotics	Erickson and Bennett (1965)

(continued)

Table 14.1 (continued)

Source	Organisms	Enzymes	Applications	Reference
	Saccharomyces sp.	Glucose oxidase	Antimicrobial	Kaur and Sekhon (2012)
		Ribonuclease	Antiviral	Lin et al. (2013)
		Sacrosidase	Congenital sucraseisomaltase deficiency	Kaur and Sekhon (2012)
Bacteria	*Bacillus subtilis*	Arginase	Antitumour	Kaur and Sekhon (2012)
		Bacilysin synthetase	Antibacterial	Torsten (2005)
		Nattokinase	Cardiovascular disease	Hsia et al. (2009)
		RNase	Antiviral	Kaur and Sekhon (2012)
		Urokinase	Blood clots	Zaitsev et al. (2010)
	Bacillus licheniformis	Bacitracin synthetase	Antibacterial	Edward and Arnold (1988)
	Bacillus brevis	Gramicidin synthetase	Antibacterial	Edward and Arnold (1988)
		Phenylalanine racemase	Antibacterial	Edward and Arnold (1988)
	Bacillus polymyxa	Peptidase	Dissolving blood clot, anti-inflammatory,	Kaur and Sekhon (2012)
	Clostridium perfringens	Collagenase	Skin ulcer	Nimni and Peacock Erie (1989)
	E. coli	Arginase	Antitumour	Huston et al. (1988)
		Asperginase	Leukaemia	Huston et al. (1988)
		Glutaminase	Leukaemia	Spiers and Wade (1976)
	Lactobacillus sp.	Urease	Nitrogen metabolism in ruminants	Banerjee and Aggarwal (2013)

(continued)

Table 14.1 (continued)

Source	Organisms	Enzymes	Applications	Reference
	Mycobacterium sp.	Superoxide dismutase	Antioxidant and anti-inflammatory	Kaur and Sekhon (2012)
	Nocardia sp.	Superoxide dismutase	Antioxidant and anti-inflammatory	Kaur and Sekhon (2012)
	Pseudomonas aeruginosa	Protease	Antibacterial	Srilakshmi et al. (2014)
	Streptomyces gulbargensis	Alkaline protease	Bio cleaning agent for washing surgical instruments	Vishalakshi et al. (2009)
	Streptomyces glaucescens	Tyrosinase	Treatment of Parkinson's disease	Kaur and Sekhon (2012)
	Streptomyces griseoloalbus	α-Galactosidase	Prevention of xenorejection	Anisha et al. (2008)
	Sulfobacillus sibiricus	Rhodanase	Cyanide poisoning	Kaur and Sekhon (2012)
	Serratia marcescens	Serratiopeptidase	Anti-inflammatory	Kaur and Sekhon (2012)
	Streptococci sp.	Staphylokinase	Thrombolytic agent	Kaur and Sekhon (2012)
		Streptokinase	Anticoagulant	Banerjee et al. (2004)
	Staphylococcus aureus	Staphylokinase	Thrombolytic agent	Kaur and Sekhon (2012)
	Vibrio proteolyticus	Vibrilase	Treatment of damaged tissue	Pal and Suresh (2016)

Serine Proteases possess a serine group in their site of action (Theron and Divol 2014). Serine proteases fall into the two broad categories, chymotrypsin-like or subtilisin-like on the basis of their structure (Madala et al. 2010). These types of proteolytic enzymes are abundant in both prokaryotes like bacteria and eukaryotes.

Microorganisms are favourable as sources of protease for a number of reasons (Singh et al. 2016; Sharma et al. 2017). The following are a list of some microorganisms including bacteria and fungi, capable of producing protease. Some bacterial species capable of producing the enzyme are *Bacillus amovivorus*, *Bacillus cereus*, *Bacillus circulans*, *B. laterosporus*, *B. licheniformis*, *B. megaterium*, *B. proteolyticus*, *B. pumilus*, *B. subtilis*, *Bacillus* sp., *Listeria monocytogenes*, *Geobacillus caldoproteolyticus*, *Geomicrobium* sp., *Lactobacillus brevis*,

L. plantarum, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas thermareum*. Some fungal species producing protease are *Aspergillus clavatus*, *A. flavus*, *A. niger*, *A. oryzae*, *A. terreus*, *A. ustus*, *Beauveria* sp., *Mucor* sp., *Penicillium chrysogenum*, *P. godlewskii*, *Penicillium* sp.

Bacterial and fungal proteases are applicable for the different diagnostic and therapeutic purposes in the medical industry (Singh et al. 2016; Srilakshmi et al. 2014; Banik and Prakash 2004). Proteases isolated from *Aspergillus oryzae* have found application as digestive aids (Rao et al. 1998). It also find application in pharmaceutical industries for treating different skin problems and injuries, wounds, burns, abscesses, carbuncles, elimination of keratin in acne, scar removal using keratinases, elimination of human callus, regeneration of epithelial cell, production of vaccines (Brandelli et al. 2010). Protease has such beneficial effects on skin, that the enzyme is added in cosmetic products for hydrolyzing the peptide bonds of elastin, collagen and degradation of keratin of the skin (Brandelli et al. 2010; Mane and Tale 2015). Collagenolytic proteases find applications in wound healing, treating retained placenta, and improving adenovirus-mediated gene therapy of cancer (Ali and Muhammad 2017; Watanabe 2004). Along with broad-spectrum antibiotics, a combination of clostridial collagenase and subtilisin is used for treating burns and wounds.

Serrapeptase, also called Serratiopeptidase is a protease, obtained from the bacterial genus *Serratia* sp., belonging to enterobacteriaceae found in the gut of silkworm. This enzyme is useful in treating pain and possesses anti-inflammatory, fibrinolytic activities capable of hydrolyzing fibrin. This protease also has enti-edemic activities. It is more effective than EDTA (ethylenediaminetetraacetic acid) in replacing arterial plaque. Serrapeptase, when used in combination with another proteolytic enzyme, Serrazime, isolated from *Aspergillus melleus* and *Aspergillus oryzae*, is capable of clearing sputum (Vaisar et al. 2007). Serrapeptase is also capable of inhibiting the release of bradykinin to lessen pain. It breaks down fibrin, facilitates healing of injury and tissue repair (Rothschild 1991; Esch and Fabian 1989). Wounds containing necrotic tissue, blood clots, fibrinis cleaned up by the application of a protease commercially available as Varidase (Streptokinase-streptodornase) (Rao et al. 1998). Proteolytic enzymes used for the treatment of different kinds of inflammation, have been reported to be without any significant side effect. A serine protease, Nattokinase obtained from *Bacillus subtilis* is used as a nutraceutical in case of cardiovascular diseases. This proteolytic enzyme is capable of reducing some factors of blood clotting related with cardiovascular diseases preventing blood coagulation and dissolving thrombus (Milner 2008). Different microbial proteolytic enzymes also possess antimicrobial properties (Srilakshmi et al. 2014).

14.5.2 Uricase

Uricase (EC 1.7.3.3) is also called as urate oxidase, Uox and the urate-oxygen oxidoreductase. It oxidizes uric acid to diureide (allantoin). Uric acid contains

2,6,8 trihydroxypurine, as a keto-enol tautomer to convert into the equivalent urate (Maples and Ronald 1988). In general, during catabolism of the purine nucleotide, uric acid comes out as an end product. But, due to the absence of uricase enzyme in higher apes and humans, uric acid gets accumulated in the blood (Merriman and Dalbeth 2011). For this reason, uric acid possibly leads to the warning sign of gout, which includes redness, severe pain and swelling in joints. It is characterized by uricemia due to the deposition of ions and salts and thus forming uric acid crystals around the joints. This problem of uric acid accumulation calls for the need of uricase enzyme which will be able to treat diseases like gout.

Microorganism, like *Escherichia coli, Bacillus pasteurii,* and *Proteus mirabilis* produce uricase within the system, while others produce uricase extracellularly like *Streptomyces albogriseolus, Candida tropicalis, Microbacterium, Pseudomonas aeruginosa, Bacillus thermocat.* These organisms can also be induced to produce substantial amount of extracellular uricase by changing definite components in the culture media (Gabison et al. 2010; Vogels and Van der Drift 1976). Uricolytic fungi which degrade uric acid are isolated from soil and belong to *Penicillium, Spondilocladium, Helminthosporium, Fusarium, Stemphylium, Aspergillus, Geotrichum, Alternaria, Curvularia* and *Mucor* genera (El Din and El-Fallal 1996; Nagger and Emara 1980). Researchers have also isolated uricolytic bacteria showing high uricolytic activity like *Lactococcus, Pseudomonas* and *Enterobacter* from gut of apple snail (*Pomacea canaliculata*). Both nitrogen and phosphorus get symbiotically recycled in the snail (Koch et al. 2014). Report states that two types of uricolysis of uric acid occur in the snail, first one is non-enzymatic forming antioxidant and second one is enzymatic forming allantoin and ammonia which could be supplied as amino acid, protein and purine (Vega et al. 2007; Giraud-Billoud et al. 2008).

In humans, urate, a product of xanthine oxidase which oxidizes xanthine is reabsorbed in the kidney after excretion. In healthy human being, when the solubility of urate exce in plasma urate level, it cause hyperuricemia, followed by lowering of plasma xanthine level. It is also associated with hyperxanthinemia which often leads to kidney dysfunction. Due to the formation of crystals it may lead to gout, chronic kidney disease and tumour lysis syndrome (Davidson et al. 2004; Edwards 2009; Johnson et al. 2009; Kang et al. 2002).

Nowadays multiple drugs are available into the market like allopurinol and febuxostat which acts on urate, urate transporter and xanthine oxidase. But due to the hypersensitivity and non- responsiveness to allopurinol in patients, this drug shows intolerance and toxicity against allopurinol (Chohan and Becker 2009; Edwards 2009; Richette and Bardin 2006; Vogt 2005). In the case of febuxostat, it is tolerated by most patients but the efficiency of febuxostat to treat gout has not been confirmed (Chohan and Becker 2009; Terkeltaub 2009). Uricase, anti-hyperuricemia acts on urate catalyze and oxidize into 5-hydroxyisourate and hydrogen peroxide (Ramazzina et al. 2006). 5-hydroxyisourate cleaves into allantoin and carbon dioxide that is highly soluble in plasma and excreted from the kidney. Thus, these evidences exhibit that microbial uricase will prove effective in treating different diseases and can be of immense help in health care sector.

14.5.3 Lipase

Lipases (E.C. 3.1.1.3) (triacylglycerol acyl hydrolases) catalyze hydrolysis of the long chain fatty acids and formation of esters. All lipases are having a α/β hydrolase fold, a catalytic triad containing a nucleophilic serine and a glutamate or aspartate residue bonded to a histidine by hydrogen bonding (Lotti 2007). Higher eukaryotes and microorganisms are capable to produce lipase and they are available in various habitats like decaying foods, vegetable oils processing industries, compost heaps, oil contaminated soil and dairies (Sztajer et al. 1988; Wang et al. 1995).

Different microorganism (bacteria, actinomycetes, yeast and fungi) produce various type of lipase having differences in their chemical, physical and biological properties. It has been seen that lipase produced by bacteria in the dairy industry contributes as flavouring agent in fermented dairy products. Microbial lipases are inductive extracellular enzymes and amalgamate within the cell and then get transported to its exterior surface. Pseudomonas bacteria like *Pseudomonas alcaligenes, Pseudomonas mendocina, Pseudomonas cepacia* are known for producing commercial lipases (Chigusa et al. 1996). Physicochemical properties of lipase are different and depend on substrate, temperature, metal ions and pH. Several methods have been established to detect the lipase activity in microbes like clear halos and opaque zones. These zones are formed in the region of the colonies with the presence of emulsified tributyrin and triolein substrate (Atlas 1996; Cardenas et al. 2001).

The Western diet contains approximately 90% triacylglycerol or triglycerides as the main fat content. Triacylglycerol contains glycerol connecting three fatty acids through ester bonds. Absorption occurs in human digestive system when these triglycerides are hydrolyzed by some intestinal enzymes like lipases. Hydrolysis of triacylglycerides releases free fatty acids and 2- monoglycerides which easily get sopped up by the duodenum (Mukherjee 2003). Obesity is a worldwide plague linked with noteworthy morbidity and mortality in all age group of people. Western people take fat rich diet and inefficiency of lipase to digest fat may cause life threatening disorders. Taking of commercially produced lipase after meal can prove to be helpful for these people. Serous gland, present at the back of tongue and secretes lingual lipase which helps to digest the fat (Birari and Bhutani 2007; Hamosh 1990). Human gastric lipase is secreted by the fundic mucosa of the stomach and the beauty of this enzyme is that it can remain stable even at low pH. Other than these, many lipase enzymes are secreted by acinar cells of pancreas namely, pancreatic lipase related-protein 1 and 2, cholesterol esterase, colipase-dependent lipase, and phospholipase A2. Pancreas also secretes another enzyme named as colipase which binds to bile acid micelles and works with pancreas lipase to emulsify lipid droplets (Van et al. 2004). In patients with pancreatitis, additional taking of lipase along with amylase and protease will be an immense helpful aid in proper digestion for them.

14.5.4 Fibrinolytic Enzyme

Thrombosis is the most leading cardiovascular disease of today's world. Treatment of thrombosis includes the effective use of fibrinolytic enzymes in drugs. Fibrinolytic enzyme takes part in fibrinolysis which breaks down the fibrin clots. The plasmin is the main fibrinolytic part which chops off the fibrin mesh of the circulating blood clot into small fragments. These fragments are further hydrolyzed by proteases. Recently, extensive studies were carried on different fibrinolytic enzymes like urokinase, tissue plasminogen activator and bacterial plasminogen activator streptokinase and these studies have showed the potential use of fibrinolytic enzymes against thrombosis. Ser221, His64, and Asp32 form the catalytic triad of fibrinolytic enzymes. It has also been documented that the enzyme activity of several fibrinolytic enzymes is influenced by the presence of DTT (Dithiothreitol) and/or Beta mercaptoethanol. They act on –SH groups in the catalytic site and shows the presence of intramolecular disulfide linkage. On the basis of catalytic mechanisms, fibrinolytic enzymes are of 3 types: Serine proteases, metalloproteases, and serine metalloprotease. Presence of a serine group in their active site is the characteristic of Serine proteases, for example, NK (Nattokinase) subtilisin DFE (Douchi Fibrinolytic Enzyme). Metalloproteases belonging to the most diverse group of proteases are characterized by the requirement of a divalent metal ion for their activity. There are also reports showing this category of fibrinolytic enzyme requires divalent metal ion, such as Zn^{2+}, Co^{2+} and Hg^{2+}. The protease activity of these enzymes is inhibited by chelating agents, such as EDTA (Ethylene diamine tetra acetic acid) and EGTA (Ethylene glycol tetra acetic acid).

Among the various available sources of fibrinolytic enzyme, microorganism is considered as dominant source due to its diversity and suitability for production of fibrinolytic enzymes in large scale. Recently, many fibrinolytic enzyme producing microbes have been extracted from both food and non-food sources. The bacteria belonging to genus *Bacillus,* isolated from conventional fermented products has been observed to be a potent candidate for fibrinolytic protease production. Nattokinase (NK) produced by *Bacillus natto* was the first screened fibrinolytic enzyme isolated from natto which is a soybean-fermented food. Consequently, traditional fermented foods from all round the world have been considered as excellent source of fibrinolytic enzyme producing microbes, which are GRAS category (Generally Regarded As Safe). Therefore, fibrinolytic enzymes, isolated from GRAS microorganisms, have the ability to develop as functional food additives. These can also be proved to be powerful drugs to cure thrombosis. Few fibrinolytic enzyme producing fungi are *Actinomycetes*, *Penicillium chrysogenum*, *Aspergillus ochraceous,* etc. and algae namely; *Codium latum* and *C. divaricatum* are also reported to produce the enzyme. Serine metalloproteases are few exceptional examples of fibrinolytic enzymes, which are produced by *Rhizopus chinensis* and Streptomyces sp.

Currently, fibrinolytic enzymes are widely used as anti-inflammatories, anticoagulants, thrombolytics, mucolytics, and antimicrobials. The formation of blood clot within blood vessel is one of the major concerns for cardiovascular

diseases. Blood clots primarily contain fibrin as the major protein component. It is derived from fibrinogen through proteolysis by thrombin. The prime functioning of fibrinolytic enzyme is to hydrolyze the fibrin clots and thereby avoid thrombosis in blood vessels. Fibrinolysis mechanism of Nattokinase (NK) has been studied more vividly and report says that NK cleaves cross-linked fibrin and activate the production of t-PA (Tissue Plasminogen Activator). t-PA converts inactive plasminogen to active plasmin. Moreover, the primary inhibitor of fibrinolysis PAI-1 (Plasminogen Activator Inhibitor Type 1) is cleaved and inactivated by NK and thus, total fibrinolytic activity is regulated by the relative ratio of PAI-1 and t-PA. Scientific studies have demonstrated the efficacy of NK in promoting in-vivo lysis of thrombi. It has been observed that a daily dose of NK taken intravenously within 12 h of a stroke has been able to dissolve clots more efficiently than that of commercially available recombinant t-PA and other drugs. A new fibrinolytic metalloprotease has been extracted from edible mushroom which have showed to treat thrombolysis with minimal side effects.

14.5.5 Chitinase

Chitinase (EC 3.2.1.14) catalyzes the hydrolysis of the β-1,4 bonds of the N-acetyl-β-D-glucosamine of chitin and chitin dextrins. It is generally bound to protein and polymeric carbohydrate molecules. As per the X-ray diffraction studies, it has been seen that chitinase has crystalline structure and is water insoluble in nature (Majeti and Kumar 2000; Roberts 1992). Three types of arrangement in chitin chain are present like α, β and γ. Generally, in fungi, insects and crustaceans, α form of chitinase is foremost and more stable and also contains substituting parallel and anti-parallel chains. In case of marine organisms, only β form is present and contains parallel chains. But the detailed studies about γ form are still pending (Roberts 1992). Two major groups of chitinases are classified as endo and exo. In case of endochitinases, the enzymes cuts at random internal sites of N-acetylglucosamine and produce soluble and low molecular oligomers of chitin like dimer diacetyl chitobiose, chito tetraose and triose. Exo-chitinases have been categorized into two parts, namely, (1) chitobiosidases (EC 3.2.1.29), which hydrolyze at the nonreducing end of the N-acetylglucosamine and releases the di-acetylchitobiose and (2) 1–4-β-N-acetylglucosaminidases (EC 3.2.1.30), which cut the chitobiosidases and endochitinases and generate monomers of chitin (Cohen-Kupiec and Chet 1998; Novotna et al. 2008).

Furthermore, two enzymes are present in mammalian chitinases, true chitinases which is enzymatically active and another is chitinase-like proteins which have chitin binding activity but no enzymatic activity. Chitotriosidase and acidic mammalian chitinase are regarded as true chitinases. Both belong to the family of glycosyl hydrolases and having an eight-stranded α/β barrel catalytic core arrangement (Boot et al. 2001; Bussink et al. 2007).

Different microorganism (bacteria and eukaryotic fungi) are capable to produce chitinases such as *Bacillus subtilis, Penicillium aculeatum, Paenibacillus*

sp. CHE-N1, *Cellulosimicrobium cellulans* FXX, *Trichoderma harzianum* TUBF 781, *Lecanicillium fungicola* and others. Fungi have the disadvantage due to the formation of mycelia at the end phase of fermentation compared to bacterial fermentation. Chitin degrading microorganisms are present in natural soil from where it can easily isolate the strain for chitinase production. Among all microorganisms, approximately 90–99%, *Streptomyces* sp. produces chitinolytic enzymes and also a better source of enzyme inhibitors, vitamins, antibiotics and immuno-modifiers (Kumar and Gupta 2006).

From ancient time, the existence of chitinase in human and their role in different disease of human are been noted (Bargagli et al. 2007; Boot et al. 1999; Chen et al. 2009; Hollak et al. 1994; Tjoelker et al. 2000; Vazquez-Torres and Balish 1997; Vicencio et al. 2008). Apart from the antimicrobial role, chitinase also helps in therapeutic treatment of cancer, Gaucher disease, decomposition of chitin and asthma. It could be taken as a simple and cost-effective biofungicide and biopesticide marker for overseeing disease progress and a goal for novel therapeutics for human health protection.

Chitotriosidases have two major isoforms which differ by molecular mass and isoelectric points. These are purified from spleen of Gaucher patients. Acidic mammalian chitinase was discovered which has acidic isoelectric point and works at optimum pH of 2 (Boot et al. 2001). Reports says that both acidic mammalian chitinase and chitotriosidase are tempting candidates for preventing infections caused by chitin containing organisms, specifically pathogenic fungi.

14.5.6 Collagenase

Collagenases (EC 3.4.24.3) are promising enzymes which has widespread application in pharmaceutical, health and food industries. Collagen is an important protein present in extracellular tissues like skin, blood vessels, cartilage and tendons. It is also the organic component of bones, teeth and cornea. One-third of the amino acids constituting the collagen are composed of three alpha-chains of glycine, proline and hydroxyproline. The repeating units of these amino acids form primary, secondary, tertiary and quaternary structure of collagen which then forms the framework of the tissue formation. It can be produced by various sources such as animal, plant and microorganisms. The common microbial source for production of collagenase is *C. perfringens, C. histolyticum* and *C. tetani.* These collagenases are capable of splitting each polypeptide chain of collagen into multiple sites. They also function in disrupting connective tissues due to the hydrolysis of collagen in the host cells (Pal and Suresh 2016). The breakdown of collagen by collagenase becomes compulsory for many physiological functioning of the development like tissue embryo development, morphogenesis, movement of morphogens, etc. Collagenase is also influential in tissue remodelling and wound healing. Studies on collagenase have witnessed its high importance in plastic surgeries where skin cells are grown on collagen backbone (Nimni and Peacock Erie 1989). But, accumulation of excessive collagen can induce malfunctioning of organs as the organ tissues become stiff with excessive

collagen formation. It has been reported by several researchers that with aging, collagen synthesis in body starts decreasing and hence, demand of collagen in skin, hair and bone tissues increases. Collagen peptides which is known for collagen degradation, holds several biological activities of food, nutritional and medical industries. These collagen degradation products could be effectively added to food and beverages to improve their functional and nutritional properties without causing any harmful effect in human body (Holmes et al. 2001).

Collagenase has the tendency to break down four types of collagen viz. collagen I, II, III, IV and also is capable of hydrolyzing the triple-helical domain of collagen under various physiological conditions (Birkedal-Hansen et al. 1988). Collagenase often finds applications in medical sector for separating tissue cells in medical investigation. These enzymes have been successfully tested in case of diabetes patients to remove and relocate the insulin gland cells. In recent times, collagenase has been employed to replace some invasive treatments for diseases where excessive collagen deposition caused disorders in physiological functioning of the body (Harrington 1996). One-third of the protein in human body is made up of collagen. Hence, a small change in its production or disruption might cause serious health issue. Overproduction of collagen in unsuitable places can be controlled by injecting collagenase intravenously or by applying ointment thus, stopping production of disruptive collagen.

14.5.7 Amylase

Amylase is a starch hydrolyzing enzyme, which hydrolyzes starch molecules to glucose. Since this enzyme acts on α-1-4 glycosidic bonds, it is called glycoside hydrolase. Amylases have been classified broadly into 3 subtypes, α, β and γ, of which, α-amylase has been found to catalyze faster than β-amylase.

α-amylase (EC 3.2.1.1) hydrolyzes internal α-1, 4-glycosidic bonds in starch (Sundarram and Thirupathihalli 2014). α-amylases are calcium metalloenzymes, depending on a metal co factor for activity (rcsb.org).

β-amylase (EC 3.2.1.2) is an exo-hydrolase. Its catalytic action is from the nonreducing end of a polysaccharide. It catalyzes the breakdown of α-1, 4-glucan bonds producing two maltose units.

γ-amylase (EC 3.2.1.3) breaks down α(1–6) glycosidic bonds, and the last α(1–4) glycosidic bonds at the nonreducing end of starch, producing glucose. The γ-amylase shows maximum activity in acidic environment.

Microbial amylases produced by bacterial and fungal species have been used in different industrial sectors like fermentation, food and pharmaceuticals and also in scientific research (Subash et al. 2017). The amount of amylase produced depends on the specific strain of the microbe and its origin, i.e. the environment from which it has been isolated. The process parameters like fermentation time, temperature, pH, sources of carbon and nitrogen affect the rate of the microbial synthesis of amylase (Gopinath et al. 2017). Amongst the different microbial species capable of producing

amylase, bacterial production of the enzyme has been found to be faster and more economic, and bacterial strains are easier to be genetically modified.

Amylase producing bacterial strains include many species of *Bacillus* like, *Bacillus amyloliquefaciens*, *B. licheniformis*, *B. cereus*, *B. vulgaris*, *B. mesentericus*, *B. coagulans*, *B. halodurans*, *B. megaterium*, *B. subtilis*, *B. polymyxa*, *B. stearothermophilus*, and from other species, like, *Caldimonas taiwanensis*, *Haloarcula hispanica*, *Chromohalobacter* sp., *Pseudomonas stutzeri*, *Corynebacterium gigantea*, *Halobacillus* sp., *Lactobacillus manihotivorans*, *Halomonas meridian, Lactobacillus fermentum*, *Rhodothermus marinus, Geobacillus thermoleovorans,* etc.

Fungal production of amylase is advantageous, as the enzyme is secreted extracellularly, and fungal species are capable of producing the enzyme via solid state fermentation by penetrating hard substrates. Amylase has been produced by fungal species like *Aspergillus awamori*, *A. kawachii*, *A. niger*, *A. flavus*, *A. fumigatus*, *A. oryzae*, *Cryptococcus flavus*, *Mucor* sp., *Penicillium brunneum*, *P. chrysogenum, P. expansum*, *P. fellutanum*, *P. janthinellum*, *P. camemberti*, *P. olsonii*, *Pycnoporus sanguineus*, *Streptomyces rimosus*, *Thermomyces lanuginosus*, and *Thermomonospora curvata*.

Amylases find uses in different industrial applications involving the food sector, pharmaceuticals, paper, detergents, textile industries (Hussain et al. 2013; de Souza and Magalhaes 2010). In the medical sector, the enzyme, α-amylase is used in the preparation of different digestive aids so that the digestibility of the food increases (Elmarzugi et al. 2014). In the presence of bacterial α-amylase, digestion of consumed food starch is better. Therefore, digestive aid containing α-amylase is prepared for the treatment of patients, whose digestive capacity decreases due to illness. Such digestive aids are commercially sold as tablet or syrup as digestive tonics (Faulks and Bailey 1990). Other clinical therapeutic applications of amylases include those in surgery. α-amylase is used in many pharmaceutical enzyme replacement preparations that are applied in treating pancreatic insufficiency.

14.6 Conclusion and Future Scope

Recent times have witnessed the use of microbial enzymes in diverse dimensions of pharmaceutical industries. Biopharmaceuticals in form of microbial enzymes are permeating in therapeutics, diagnostics, drug discovery and tissue engineering. Enzymes are being used in diagnosis of various diseases and further development in sensitivity and cost of the detection of these diseases can be expected. Growth in therapeutic applications in form of pills, ointments and injections has also been witnessed. The vivid understanding of the microbial enzymes and their use in treatment of the diseases at molecular level may aid in enhancing the efficiency of the enzyme therapy. Breakthrough advancement in purification of the microbial enzymes from various incompatible contaminants in industrial scale is highly expected. Therapeutic enzymes in gastrointestinal diseases are in tremendous demand and presently, concoction of enzymes is taken as supplements along with

food. Voluminous studies have been conducted to utilize the varied microbial resources from exotic locations all over the world. Medically relevant microbial enzymes are in rampant use as antimicrobial, anticoagulants, anti-inflammatories, thrombolytics, fibrinolytics and digestive aids. Antibiotic resistant diseases can be ameliorated by implementing antibiotic coupled with enzyme therapy. A hefty amount of scope is available to explore in antibiotic enzyme coupled therapy. Thus, this chapter concludes that there is need to dig out information on microbes and their secretions, so that these can be implemented in improving human health.

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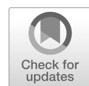

15 Significance of Enzymes in Modern Healthcare: From Diagnosis to Therapy

Pragyan Mishra, Shibangini Beura, and Rahul Modak

Abstract

The human genome encodes around 25,000–30,000 genes, translating to many more proteins resulting from alternate splicing. A number of these proteins are enzymes possessing regulatory function in cellular and biochemical processes. Deregulation of enzyme activity can alter cell fate, often leading to diseased state, thereby presenting an opportunity for therapeutic interventions. Presently, many of such enzymes have been established as targets for manufacturing drugs (natural or synthetic) that will alter their activity. Specific enzyme activities reflect diverse functionality of our body thus, providing vital information about overall health of an individual. Pathogen, especially virus detection is often facilitated by evaluation of specific enzyme activities. High specificity, low immunogenicity, high stability, and greater consistency of enzymes have opened up to serve as potential alternative target for therapeutic intervention. Advances in recombinant technology and protein engineering have evolved enzymes with wide implications in disease diagnosis and pharmacological drug synthesis using enzyme-based immunoassays and green chemistry, respectively. Taken together enzymes play central and all around role in healthcare and disease diagnostics. In the present chapter, we will brief upon well-established enzymes in healthcare thereby highlighting upon new therapeutic targets and novel methodologies adapted as emerging therapies for existing as well as "incurable" diseases.

Keywords

Enzyme therapy · Diagnostics · Anticancer drugs · Epigenetic therapy · Drug delivery · Enzyme inhibitors

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15.1 Introduction

In this era of modernisation and development, where everyone is actively involved in accomplishment of their respective goals, lack of time and abrupt lifestyle has turned health care into a major concern. Five major dimensions influencing human health are physical, intellectual, environmental, sociocultural, and emotional. The major risk factors influencing human health are age, lifestyle, health habits, genetic constraints, physiological, and environmental factors. Every aspect of our daily life is dependent on proper functioning and interplay between different enzymes, deregulation of which leads to diseases. Enzymes can be the key to improve the manufacture of other medications for treating multiple diseases. Effectiveness of the drug depends upon the individual pharmacokinetics and pharmacodynamics that in turn is regulated by several enzymes. Administration of enzyme and enzyme-catalyzed products are important for clinically used drugs.

The risk of developing diseases is multifactorial. The emergence of personalised medicine i.e., treatment based on genetic differences of the individual might help in optimising therapy decisions. This would help in providing information about possible response to chemotherapeutic intervention. Such a scenario demands the development of prognostically and diagnostically relevant biomarkers and more efficient therapeutic strategies for productive and methodological treatment that can be utilised by the entire commensal population. Enzymes have a widespread role in metabolism, signaling pathways, immune responses, defense mechanisms, etc. A potential aspect of their functionality that needs to be explored further is their use as therapeutic targets. Techniques like enzyme replacement therapy and adjunct therapy are also being used for combating heterogeneity of complex diseases. However, a more depth understanding is required to develop more specific molecular targets and their inhibitors.

Enzymes have critical roles in metabolic processes of all living organisms including microbes, offering wide range of applications for diagnostic purposes. Recent advances have discovered enzymes as a potential link to prevent metabolic disorders. Different isoforms of an enzyme are structured together as fusion proteins and are used in clinical examinations as biomarkers of complex diseases. The significant increase of these isozyme levels in the serum creates possibility of the former to serve as diagnostic tool at initiation and progression of the disease. These advances have immensely shaped histopathological diagnosis and clinical intervention for several diseases. Emerging classes of enzymes such as DNA and protein methyltransferases and demethylases, kinases, N-acetyltransferases, deacetylases, crotonylases, etc., responsible for protein posttranslational modifications, play a pivotal role in diseases like glioma, artherosclerosis, Alzheimer's, cancer, etc. These enzymes function in an interdependent manner to alter gene expression and protein function in disease-specific manner. Advancement in new technologies like, high throughput whole genome and transcriptome sequencing are getting incorporated in healthcare for better understanding of genomic and epigenomic regulations during disease progression. Ultimate goal of using enzymes as

biomarkers revolves around the idea of personalised treatment based on individual's genotype and genetic profiles.

Besides diagnostics and therapeutics, there is an increasing use of enzymes as biocatalysts in the production of pharmacologically relevant drugs. They catalyze biochemical modification of xenobiotics and endogeneous chemicals. Enzymes as biocatalysts render several advantages over the chemical reactions for the synthesis of these drugs: (a) Increase in yield and specificity of the product, (b) High regio, chemo, and stereoselectivity, (c) Enhanced enantiomeric purity, (d) reduction of use of harsh chemicals and (e) reduction in cost of raw materials and waste generation. Genetically engineered combination of recombinant enzymes with high activity, stability and longer half-life is required for process optimisation and scale up. Table 15.1 represents some of the commonly used enzymes by pharma industry. Taken together enzymes play critical role in all aspects of healthcare.

15.2 Modes of Action of Enzyme-Targeted Drugs

Among the FDA-approved marketed drugs, 65% molecules harbor a substrate-related structural motif that undergoes chemical reaction within the target enzyme active site. Some of the enzyme targets are irreversibly inhibited through covalent modifications formed by the drug, where as in other cases drugs are reversible tight binding inhibitors of target enzymes. In addition to these, transition state inhibitors and entrapment of enzyme–substrate intermediates are also different methods of targeting enzyme mechanisms and opportunities for drug designing (Table 15.2).

15.2.1 Structural Analog to Enzyme Substrate

Most of the enzyme targeted drugs are substrate analogs like inhibitors, that contain a substrate related structural motif and binds to enzyme active site. Some of these classes of inhibitors also react with certain enzyme cofactors. Many antibiotics, such as carbenapem, penicillin, cephalosporin, etc. undergo catalysis by their target enzymes by acylating serine at the active site of D-Ala-D-Ala carboxypeptidase. They bear structural similarity to the bacterial peptidoglycan and hence function as substrate inhibitors. In addition to these, β-lactamase inhibitors acylate serine at active site of β-lactamase, thereby helping overcome resistance to β-lactamase.

There are purine-based and pyrimidine-based inhibitors, prescribed as anticancer drugs. They contain their related core structures targeting phosphodiesterases, RNA and DNA polymerases, adenosine deaminase, etc. HMG-CoA (β-Hydroxy β-methylglutaryl-CoA) reductase inhibitors bear HMG-like moieties that binds to the HMG binding pocket of HMG-CoA, inhibiting its activity (Istvan and Deisenhofer 2001). These group of inhibitors are broadly called as statins and often prescribed as cholesterol lowering drugs.

Table 15.1 Commonly used enzymes used in pharma industry

Enzyme	Source	Reaction	Use
γ-lactamase	*Sulfolobus* strain	γ-Lactam → γ-bicyclic lactam (2-azabicyclohept-5-en-3 one)	Intermediate of anti-HIV drug, Abacavir (Taylor et al. 1993)
Alanine: Glyoxylate transaminase	*S. solfataricus*	L-serine + Pyruvate → 3-hydroxypyruvate + Alanine	Pharmaceutical drug intermediates (Sayer et al. 2012)
L-haloacid dehalogenase	*Sulfolobus tokodaii*	Production of Chiral Halo carboxylic acid	Bioremediation, intermediate in chemical industries (Rye et al. 2007)
Carboxylesterase	*Ophiostoma novo-ulmi*	Hydrolysis and esterification	Production of pain killers (Isupov et al. 2004) Example: Naproxen
L-aminocyclase		Resolution of amino acid and its analogues	Screening for substrate specificity (Littlechild 2015)
Alcohol dehydrogenase	*Aeropyrum pernix*		Chiral alcohol production (Guy et al. 2003)
Phosphotriesterae lactonase (PLL)	*S. solfataricus* and *S. acidocaldarius*	Cleavage of lactone rings	Quorum sensing (Afriat et al. 2006; Porzio et al. 2007; Merone et al. 2008)
Bryostatin-1	*Bugula neritina*	Prokinase C agonist	Promotes synaptogenesis (Gentile and Liuzzi 2017)
R-selective transaminase	*Arthrobacter* sp.	Prositagliptin → Sitagliptin	Treatment of type II diabetes (Savile et al. 2010)
Monoamine oxidase		Synthesis of bocoprevir	Treatment of hepatitis-C infection (Choi et al. 2015; Kjellin et al. 2018)
Lipase B	*Candida antarctica*	Production of S-S-reboxetine succinate	Treatment of fibromyalgia (Hayes et al. 2011)
Lipolase		Neuroactive drug	Treatment of epilepsy and social phobia (Martinez et al. 2008)
Luizym and nortase	*Aspergillus oryzae*		Lytic enzyme deficiency syndrome

Table 15.2 Various enzyme targeting drugs with different mechanism of action

Inhibitors	Target
Structural analogues	
Antibiotics (penicillin, cephalosporin, carbenapem)	Serine type D-Ala-D-Ala carboxypeptidase
Purine and pyrimidine-based inhibitors	DNA and RNA polymerases, phophodiesterases, ribonucleoside-diphosphate reductase, adenosine deaminase, IMP dehydrogenase, xanthine oxidase, thymidine synthase
Sulfonamide inhibitors	*p*-aminobenzoic acid
Naphthoquinones	Vitamin K-epoxide reductase
Phosphate mimetics	Farnesyl-diphosphate-farnesyltransferase
Transition-state analogues	
Pentostatin	Adenosine deaminase
Captopril	Peptidyl-dipeptidase A (ACE)
Saqwnavir	HIV retropepsin
Immucillin-H	Purine nucleoside phosphorylase
Irreversible inhibitors	
β-lactam antibiotics	Serine type D-Ala-D-Ala carboxypeptidase
Anticholinesterase agents (Pyridostigmine)	Acetylcholinesterase
Fosfomycin	UDP-N-acetylglucosamine-1-carboxysinyltransferase
Aspirin	Prostaglandin endoperoxide synthase/cox
Selegiline	Mitochondrial amine oxidase
Fluxuridine	Thymidine synthase
α-difluromethylornithine	Ornithine decarboxylase
D-cycloserine	Alanine racemase
H^+/K^+ ATPase inhibitors (Omeprazole, Esomeprazole, Lansoprazole)	H^+/K^+ ATPase
Orlistat/Tetrahydrolipstatin	Triacylglycerol lipase
Gemcitabine	Ribonucleoside-diphosphate reductase
Propylthio-uracil and methimazole	Iodide peroxidase
Propyl thiouracil	Type I deiodinase
Heparin	Thrombin
Nitisinone	Hydroxy-phenylpyruvate
Warfarin	Vitamin K-epoxide reductase
Mupirocin	Aminoacyl-tRNA synthetases
Finasteride	3-oxo-5-α-steroid 4-dehydrogenase
Isoniazid	Enoyl-acyl carrier protein reductase
Allupurinol	Xanthine oxidase
Reaction intermediate traps	
Mycophenolic acid	Inosine monophosphate dehydrogenase
Fluoroquinoline antibiotics and antitumor agents (Topotecan and Innotecan)	DNA topoisomerases
Bortezomib	Prevents proteasomal degradation
Nevirapine	Non-nucleoside reverse transcriptase inhibitors

15.2.2 Transition State Inhibitors

The transition state enzyme inhibitors are popular tight binding inhibitors. There are many natural and synthetic products that have become predominant transition state inhibitors. *Streptomyces* produces pentostatin that binds to human erythrocyte enzyme adenosine deaminase, thus acting as an anticancer chemotherapeutic agent. Similarly, another transition state analogue captopril, whose structural design was based on carboxypeptidase A by-product analogue inhibitor, inhibits peptidyl dipeptidase A. Two potential transition state analogues were designed for enzyme exo-α-sialidase by structural analysis and kinetic isotope effects. They are zanamivir and oseltamivir, which are used for influenza treatment (von Itzstein et al. 1993).

From the knowledge of enzyme transition states, occurring due to enzyme ligand interactions, multiple potential transition state inhibitors can be designed. Kinetic isotope effects (KIEs) are used to study the kinetic information about transition states binding. The use of transition state analogues or inhibitors in anticancer therapy has become quite frequent and widespread. One such case is of T-cell leukemia, where Immucillin-H, a purine nucleoside phosphorylase inhibitor is in Phase IIa clinical trials (Evans et al. 2004). The lack of enzyme PNPase confers to specific T-cell immune-deficiency and cancer. Inhibitors against this can suppress the T-cell auto-immune disorder as well as T-cell leukemia. Immucillin-H and Immucillin-G were synthesised in context of bovine PNPase. Immucillin-H has low affinity for the human PNPase, hence a new inhibitor DADMe-Immunicillin-H mimicking the human PNPase transition state structure were synthesised. Currently, DADMe-Immunicillin-H is in Phase I clinical trials (Lewandowicz et al. 2003).

The enzyme methyl thio adenosine phosphorylase (MTAP) produces metabolites leading to *S*-adenosyl methionine (SAM). SAM in turn results in the production of polyamines which is known to promote cell proliferation. Transition state inhibitors, MT-DADMe-ImmA, is a human MTAP inhibitor, showing remission of head and neck cancer in mice. This molecule is undergoing preclinical trials against cancer (Basu et al. 2007).

5-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase (MTAN), in bacterial polyamine pathway uses *S*-adenosylhomocysteine (SAH) and 5-′-methylthioadenosine as substrates. *S*-ribosylhomocysteine is a product of SAH hydrolysis by MTAN and this is a precursor product of quorum sensing molecules involved in biofilm formation leading to antibiotic resistance. P-ClPhT-DADMe-immucillin A, a potent MTAN inhibitor, is under preclinical trials as antibacterial agent against *E. coli* (Singh et al. 2005). The use of MTAN inhibitors will pave new insights in dealing with antibiotic resistance by targeting quorum sensing pathways in bacteria.

15.2.3 Irreversible Enzyme Inhibitors

Many irreversible enzyme inhibitors covalently modify enzymes upon binding. The drug is tightly bound to the enzyme and the extent of binding varies from even hours

to days. Enzyme inhibitors of enoyl-acyl carrier protein reductase and 3-oxo-5-α-steroid 4-dehydrogenase form covalent complexes with the substrate and irreversible inhibit the enzyme action.

The β-lactam antibiotics are a group of irreversible inhibitors used for inactivating serine type D-Ala-D-Ala carboxypeptidase by acylating serine present in the active site of the enzyme. This results in blocking the bacterial transpeptidase activity aiding to decreased virulence. But, bacteria also confer resistance to these group of antibiotics by β-lactamase, that hydrolyses β-lactams. Therefore, tazobactam, clavulanate, and sulbactam, naturally occurring β-lactams are used to overcome the resistance. These group of β-lactams form stable intermediates and inactivate carboxypeptidase (Christensen et al. 1990). Another of such serine hydrolase, acetylcholinesterase inhibitor pyridostigmine attacks the enzyme active site serine by acting as an alternate substrate. This leads to the active site serine carbamoylation, and these intermediates mediate inhibition till 4 h.

Fosfomycin, an antibiotic discovered from *Streptomyces*, is a phosphoenolpyruvate (PEP) substrate analogue and covalently acrylates cysteine present in the active site of VDP-N-acetylglucosamine 1-carboxyvinyltransferase (Thomas et al. 2004). Cyclooxygenase (COX) or prostaglandin endoperoxide synthase has a potent irreversible inhibitor known as aspirin which is a very popular drug worldwide. This drug functions similarly by covalently modifying the serine residues and forming a compound, Hydroxy-acetyl serine, thereby inactivating the enzyme. Unspecific mono-oxygenase also known as aromatase that converts androgens to estrogens, is inhibited by exemestane, establishing aromatase for targeted antiestrogen therapy.

There are several other inhibitors which have shown potential in inactivating the enzymes and are even marketed. The H^+/K^+ ATPase inhibitors are one of many such drugs. Lansoprazole, omeprazole, and esmoprazole are marketed to inhibit gastric cells H^+/K^+ ATPase by reacting with cysteines present on its extracellular loops (Robertson 2005). Anticoagulants are a class of drugs that inactivates clot formation. Heparin, the popular and widely used anticoagulant accelerates the reaction to 10,000-fold by acting on thrombin-antithrombin serine proteases (Rezaie and Olson 2000).

15.2.4 Reaction Intermediate Traps

Another mechanism of targeting the enzyme includes reaction intermediate trapping that occurs by forming covalent inhibitory complex. DNA Topoisomerase and inosine monophosphate dehydrogenase are two such enzymes for drug targets. DNA Topoisomerases present in both mammals and bacteria catalyse the rearrangement of DNA. Irinotecan and Topotecan (antitumor agents), alongside fluoroquinoline antibiotics are used to target DNA Toposiomerase (Higgins et al. 2003). In case of Topoisomerase I, camptothecin binds near DNA cleavage site and stabilises the formed covalent complex. But in case of Topoisomerase II, the fluoroquinoline antibiotics are used to stabilise the intermediate complex (Redinbo

et al. 1998). This is due to the difference in mechanism of action of both the topoisomerase enzymes.

Other class of enzyme based drugs such as noncompetitive inhibitors are also available that do not bind to enzyme active site, but to the allosteric site thereby slowing the rate of enzyme catalysis. One of such inhibitor is nevirapine which is a non-nucleoside reverse transcriptase inhibitor that functions by binding to the enzyme allosteric site. Hence, inhibitors that slow down chemical catalysis more are capable of becoming potent drugs.

15.3 Enzymes as Therapeutic Targets

The development of effective enzyme inhibitors is based on target identification and enzyme catalysis. It takes quite a long time for new drug designing, to its discovery and lastly being tested, approved by the FDA and other regulatory bodies for clinical application. Traditionally nuclear hormone receptors, ion channels, G-protein coupled receptors, enzymes, transporters, and nucleic acid metabolism pathways have been targeted to regulate cellular responses and thus for drug development. In recent past, some new enzymes have been targeted to develop clinically approved drugs.

15.3.1 Renin

In humans, blood pressure is maintained by the renin-angiotensin system, providing an important area for developing antihypertensive drugs. In the renin-angiotensin pathway, angiotensinogen is converted to angiotensin I by renin, an aspartyl protease. Angiotensin I is further converted to angiotensin II by angiotensin converting enzyme (ACE). Angiotensin II is the active enzyme that binds to angiotensin receptor, and inhibitors against this receptor as well as ACE are effective during hypertensive conditions by lowering the blood pressure. Additionally, these inhibitors also result in release of renin to plasma during the process. In 2007, aliskiren became the first renin inhibitor and first antihypertensive drug to be approved and marketed by the FDA (Wood et al. 2003). This renin inhibitor is a hydroxyl ethylene transition state inhibitor containing a hydrophobic moiety at the C-terminal that binds near renin active site binding pocket (Gradman et al. 2005).

15.3.2 Protein Kinases

Different catalytic activities of protein tyrosine kinases are performed by two classes of proteins, i.e. receptor protein tyrosine kinases and nonspecific protein tyrosine kinases. The nonspecific protein tyrosine kinases do not contain a transmembrane protein domain, whereas receptor protein tyrosine kinase harbors a transmembrane protein domain (Robinson et al. 2000). Till now, 58 and 32 receptor protein tyrosine kinases and nonspecific protein tyrosine kinases respectively have been identified in

the human genome. The receptor protein tyrosine kinases bind to ligands, undergo dimerisation and internalisation, which are absent in non-specific protein tyrosine kinases. Drugs targeting specific protein tyrosine kinase targets have been developed. All the protein tyrosine kinase inhibitors available in market function as reversible inhibitors and show efficacy against tumor suppression, but due to mutations in the enzyme they rapidly develop drug resistance.

Recently, there is evidence to show that irreversible inhibitors have an advantage in circumventing the acquired drug resistance over previously used reversible inhibitors (Kwak et al. 2005). The advantage of irreversible kinase inhibitors over reversible kinase inhibitors is its maintained inhibition throughout. In case of the later, there is speculation that inhibitors might dissociate during dimerisation or internalisation, leading to normal signal transduction. Mitogen activated protein kinase (MAPK), a serine/threonine protein kinase and mTor kinase are two other kinases established as enzyme targets. Sorafenib, an inhibitor of p38 enzyme belonging to MAPK pathway, is prescribed as anticancer drug. mTor (mammalian target of rapamycin) protein harbors multiple domains including an integral mTor kinase domain and FKBP-12 rapamycin binding domain (Yonezawa 2005). A derivative of rapamycin, Temsirolimus, modulates mTor signaling pathway by binding to FKBP-12 domain, leading to altered substrate presentation to the kinase domain. This binding results in decreased phosphorylation and reduced mTor signalling.

15.3.3 Leukotriene Inhibitors

Leukotrienes act as immune regulating mediators having potential pathogenic role in respiratory tract diseases such as asthma and hay fever by eliciting inflammatory and allergic reactions (Araújo et al. 2018). Cys LTs, known as cysteinyl leukotrienes also contribute to Alzheimer's disease and upon administration of Cys LT1 antagonist in rats, elevated neurogenesis, improved memory and decreased neural inflammation was observed (Marschallinger et al. 2015). Cys LT1 antagonist, montelukast is also effective against asthma and part of certain cough suppressing syrups. Zileuton, a 5-LOX inhibitor is marketed in the US for the treatment against asthma after showing promising results against different inflammatory diseases (Steinhilber and Hofmann 2014).

LTA4H, one of the downstream enzymes that interacts with 5-LOX during leukotriene biosynthesis has been used to develop inhibitors for use during pulmonary arterial hypertension and neoplasia (Bhatt et al. 2017). Bestatin is targeted to be used for pulmonary arterial hypertension and tosedostat is synthesised for use against variety of cancers acting as an antineoplastic drug. These two inhibitors are currently into clinical trials.

15.3.4 Dipeptidyl Peptidase IV (DPP IV)

Glucagon-like peptide (GLP) stimulates the release of insulin from pancreas, but plasma protein DPP IV hydrolyses and inactivates GLP-1. Hence, DPP IV inhibitors can be beneficial in case of type II diabetes, by promoting longer half-life of GLP-1 and sustained insulin release. Following this hypothesis, during glucose challenge DPP IV inhibitors promoted longer half-life for GLP-1 and reduced plasma glucose. Such DPP IV inhibitors are boronic acid and nitrile acid based, in which the nitrile and boronic acid groups interact with DPP IV active site serine resulting in DPP IV inhibition.

15.4 Enzymes as Drugs (Enzyme Therapy)

Therapeutic enzymes have been in use for quite a long time and are often considered under replacement therapies for different diseases. They serve as better therapeutic agents due to their affinity and high specificity towards target substrates. The most commonly used therapeutic enzymes are pepsin, β-amylase and lipase used in various combinations to treat common digestive disorders.

Activase, the first recombinant enzyme used as drug is a tissue plasminogen activator and has been approved by the FDA since 1990s. This enzyme commonly known as clot buster, is used to treat patients having heart attack due to a clot causing arterial blockage. Since then many such enzyme anticoagulants have been approved by FDA. Adagen, another drug enzyme during the 1990s, was approved by the FDA for the treatment of patients afflicted with severe combined immune-deficiency (SCID) disease. It is a form of adenosine deaminase (ADA) of bovine origin. Acute deficiency of endogeneous ADA causes accumulation of adenosine in body, leading to SCID. This excess circulating adenosine in patients is cleaved by administering bovine ADA, leading to reduced toxicity arising because of high adenosine levels. To increase the efficiency of the enzyme during treatment, ADA is treated with polyethylene glycol (PEG) that enhances the enzyme half-life. This modification also decreases its immunological reaction as the drug is of bovine origin (Roberts et al. 2012). The approval of these two enzyme drugs, Activase and Adagen by the FDA, initiated a new era for the use of enzymes as therapeutic drugs.

15.4.1 Lysosome Storage Disorders

In Gaucher disease, there is increased accumulation of glucocerebroside inside the cells due to the deficiency of glucocerebrosidase. In the form of glucerase injection, ceredase (placental glucocerebrosidase) is administered to patients, and targeted to cellular compartments having elevated levels of glucocerebroside. This is an instance of enzyme replacement therapy, where the deficient glucocerebrosidase role is carried out exogenously by the supplemented enzyme. In case of other lysosome storage disorders (LSDs) such as the Fabry’s disease, there is deficiency

of enzyme α-galactosidase, that leads to the accumulation of glycolipids within blood vessels resulting in impairment of normal function of body organs (Germain 2002). Hence, this genetic disease is termed as fat storage disorder. Recombinant α-galactosidase enzymes expressed in human and CHO cells, have completed the clinical trials and are approved by the FDA.

Enzyme replacement therapy is considered as a treatment for other forms of lysosome storage disorders (LSDs) such as muco-polysaccharide storage disorder (MPS) (Kakkis 2002). These MPS VI and MPS II are genetic disorders resulting from the malfunctioning or deficiency of lysosomal enzymes in the cell that break-down glycosanimoglycans into simple molecules. In MPS, there is deficiency of α-L-iduronidase and aldurazyme after completing phase III clinical trials is used as an enzyme replacement therapy (Kakkis et al. 2001). For Monoteaux–lamy syndrome (MPS VI), a recombinant N-acetyl galactosamine-4-sulfatase, known as Aryplase has successfully completed the clinical trials and is approved and marketed by the FDA. Hunter's disease (MPS II) is another form of MPS that occurs due to the deficiency of iduronate-2-sulfatase (I2S) leading to deposition of heparin and dermatan sulfate in body tissues. The enzyme replacement therapy for MPS II has passed Phase I/II clinical trials showing a dose-dependent decrease in GAG (glycosaminoglycan). Due to deficiency of lysosomal α-glucosidase, there is accumulation of lysosomal glycogen resulting in glycogen storage disease type II, also known as Pompe disease. This disorder causes damage to both nerve and muscle cells in the body. Recombinant enzymes have shown promising preliminary results for therapeutic purposes (Koeberl et al. 2007).

15.4.2 Oral Enzyme Therapy

Instead of providing intravenous injection, oral administration of enzyme formulations is used to treat several diseases. In congenital sucrose-isomaltase deficiency (CSID), disaccharide sucrose remains unutilised. To treat this condition, sacrosidase (β-fructofuranoside fructohydrolase) is isolated from *Saccharomyces cerevisiae* and administered orally to hydrolyse sucrose allowing CSID patients to consume a normal diet.

Deficiency or mutation in the phenylalanine hydroxylase enzyme that converts phenylalanine to tyrosine causes phenylketonuria (PKU). Oral administration of phenylase supplements the deficiency of this enzyme. Recombinant PAL (phenylalanine ammonia lyase) from yeast has been shown to catalyse the enzymatic degradation of phenylalanine. In case of cystic fibrosis patients, a condition termed as pancreatic insufficiency is observed and pancreatic enzyme mixture of amylases, lipases and proteases is administered to treat the condition. This enzyme mixture is also used to treat fat mal-absorption in HIV patients. In addition to this, there is another pancreatic enzyme mixture marketed by FDA under the name TheraCLEC Total. For other digestive diseases, such as Celiac Sprue, affecting the small intestine, peptidase supplement therapy can be administered (Shan et al. 2002). On another instance, apart from oral ingestive enzyme supplements, inhalable

Table 15.3 FDA-approved enzyme drugs for various diseases

Enzyme	Disease/disorder	Mode of delivery	Reference
Activase	Clot buster heart attacks	Intravenous	Hershfield (1995)
Adagen	SCID	Intramuscular	Hershfield (1995)
Ceredase	Gaucher disease (LSD)	Intravenous	Barton et al. (1991)
Phenylase	Phenyl ketonuria	Subcutaneous	Sarkissian et al. (1999)
TheraCLEC Total	Cystic fibrosis	Oral	Shan et al. (2002)
Oncaspar (pegaspargase)	Acute lymphoblastic leukemia	Intravenous/ Intramuscular	Avramis et al. (2002)
Dornase-α	Cystic fibrosis	Inhalation	Barton et al. (1991), Van den Hout et al. (2001)
Asparaginase	Leukemia	Intramuscular/ Intravenous	Barton et al. (1991), Van den Hout et al. (2001)
α-galactosidase A	Fabry's disease	Intravenous	Barton et al. (1991), Van den Hout et al. (2001)
PEGylated arginine deaminase	Invasive malignant melanoma	Intravenous	Sarkissian et al. (1999), Shan et al. (2002)
Acid alpha-glucosidase	Pompe disease	Intravenous	Van den Hout et al. (2001)
PEGylated urate oxidase	Gout	Intravenous	Van den Hout et al. (2001)
Hyaluronidase	Adjuvant for absorption injected drugs	Intravenous/ Intramuscular	Van den Hout et al. (2001)
Liprotamase	Pancreatic insufficiency	Oral	

formulations have also been granted approval by the FDA. Pulmozyme, marketed by the FDA, is being used in the treatment of cystic fibrosis. This enzyme acts by liquefying the mucus accumulated in lungs of cystic fibrosis patients. Pulmonary tissue damage is also diminished upon administration of pulmozyme, by reducing the level of MMPs in bronchoalveolar fluid (Ratjen et al. 2002).

In the field of cancer research, Oncaspar shows more effective and better results against acute lymphoblastic leukemia (ALL) than the already used L-asparaginase (Avramis et al. 2002). Another aspect of enzyme targeted therapy for cancer treatment, is a strategy termed as antibody directed enzyme prodrug therapy (ADEPT) and humanised monoclonal antibodies, which will be discussed in the upcoming section. Several other FDA-approved marketed drugs for different diseases listed in Table 15.3.

15.5 Antineoplastic Drugs

15.5.1 L-asparginase and mTor Inhibitors in Leukemia

Asparagine, being a non-essential amino acid is synthesised by endogenous L-asparaginase mediated biocatalysis in non-cancerous cells. Cancerous cells lack endogenous L-asparagine which is of utmost importance for their survival. Thus, scavenging the entire supply of L-asparagine from exogenous host cells and tissue is their ultimate alternative. High metabolic rates of tumor cells result in fast utilisation of L-aspargine leaving non-cancerous cells scarce of the former resulting in starvation and consequently cell death. L-asparaginase is an aminohydrolase that catalyzes the deamination of Asparagine into Ammonia and Aspartate. It has two isozymes (Batool et al. 2016): Type I is characterised for synthesis of L-asparagine and L-glutamine. Type II displays higher tumor specificity. It is used in the treatment of Acute Lymphoblastic Leukemia (ALL). Amidst numerous characterised enzymes, L-asparaginase is one of the few potential candidates that carry the ability to move forward in clinical trial and drug development. Apart from catalysis of asparagines, it displays promising antineoplastic activity (Aghaeepoor et al. 2018). Literature conveys the readily available commercial forms of asparaginase already in action: Colapase, Crasnitin, Crisantas, Pasum, Kridolase, Elspar, Erwinaze, Pegaspargasum. It is used in the treatment of Acute myeloid Leukemia (AML) and Acute Lymphoblastic leukemia (ALL). Combinatorial therapies with other drugs can be used in treatment of Hodgin's disease, lymphosarcoma, reticulosarcoma, and melanosarcoma. L-asparaginase is also used in food industry and as biosensor.

Despite of a wide range of applications, there have been rigorous attempts to improvise the existing forms of L-asparaginase due to sublethal side effects: edema, fever, hepatic dysfunction, diabetes, leucopenia, neurological seizures, hemorrhage, hypersensitivity, anaphylactic shock, immunogenicity, depression, lethargy etc.

Table 15.4 Modifications of asparginase to improve target specificity

Modifications	Advantages
• PEGylated L-asparaginase	Reduced immunoreactivity in ALL (Acute Lymphoblastic Leukemia)
	Increased thermal stability
• Asparaginase conjugated with Malic anhydride and dextran	Resistance to proteolytic digestion
	Prolonged half-life
	Retention of catalytic activity
• Poly DL-Alanyl peptides conjugated asparaginase	Enhanced therapeutic activity
	Decreased cross reactivity
	Biodegradable
• Erythrocyte/liposome mediated delivery	Compatible for drug encapsulation
	Increased life span

Table 15.4 mentions the modifications that L-ASNase has undergone to reduce the side effects and increase target specificity.

The greatest disadvantage of L-asparaginase is its unwanted glutaminase activity which results in additional severities like Pancreatitis and Coagulation Dysfunction (Aghaeepoor et al. 2018). Bioinformatics-based in silico methods were used to generate mutants of L-ASNase and verify the efficacy of each mutant in deletion of glutaminase activity. Therefore, knowing specific substrates of L-ASNase is of unmet importance. The possible hits generated by bioinformatics analysis can then be validated for new and pro-efficient anticancer drug design.

mTOR (Mammalian target of rapamycin) acts as a central processor of both intracellular and extracellular signals through regulation of many fundamental processes like metabolism, growth, proliferation and survival. It is a 289 kDa serine/threonine kinase belonging to phosphatidyl inositol Kinase (PI3K) related kinase. mTOR signaling regulate mRNA translation, cellular metabolism and energy homeostasis. Inhibition of AMPK results in reduction of total cellular ATP content and promotes mTOR expression. Activation of AMPK through by-products of p53 target genes; Sestrin1 and Sestrin2 leads to the induction of pTEN and TSC2 transcription which then downregulates S6K1 activity leading to dephosphorylation of 4E-BP1, that in turn reduces the functionality of mTORC1. This negative regulation of mTOR induces 4E-PP1 phosphorylation affecting mitochondrial stability and thus enhancing p53 mediated apoptosis. Deregulation of PI3/Akt/mTORC1 signaling leads to leukomogenesis and also have role in several other cancers. mTOR functionality in homeostasis is preserved in normal cell lines whereas needs to be regulated in cancer so as to develop more specific therapeutic strategies. We can overcome the increasing resistance of tumor cells by co-exposure to a combination of inhibitors. First generation mTOR inhibitors include rapamycin and rapalogs that might have improved effect on cancer cells if used in combination with Akt/PI3 inhibitors. Dual inhibitors are second generation inhibitors against mTORC1 that target ATP binding sites and block the phosphorylation of the signaling cascade. Despite of unsatisfying results in clinical trials, NVP-BEZ235, a small molecule inhibitor of PI3k/mTOR has shown promising antileukemic activity in the patients suffering from tumor relapse. Frequent relapse of B-ALL and T-ALL might be due to the constitutively expressed hyper active state of PI3/Akt/mTOR pathway. Further development of small molecule inhibitors in combination with other therapeutic anticancer drugs might enlighten us to overcome the increasing resistance to existing treatments.

15.5.2 HER2 Inhibitors in Breast Cancer

Breast cancer is a common form of malignancy across women worldwide, accounting for 30% of patient more than 65 years of age (Ferlay et al. 2015). This transformation to a cancerous phenotype is presented by the hormone receptor (HR) and human epidermal growth factor 2 receptor (HER2) positive marks (Jenkins et al. 2014). Activation of HER2 receptor, a tyrosine kinase receptor in breast cancer

cells, regulates genes associated with cell proliferation and survival (Gutierrez and Schiff 2011). Therefore, HER2 can be a good therapeutic target for breast cancer patients. Trastuzumab is the first HER2 targeting drug introduced for treatment of breast cancer, followed by four more HER2 inhibitors. Trastuzumab is a humanised monoclonal antibody (MAb) that targets HER2 extracellular domain, leading to HER2 internalisation and tyrosine kinase ubiquitin ligase c mediated degradation. Trastuzumab also inhibits dimerisation of HER2 and blocks Src tyrosine kinase signaling, contributing to cell cycle arrest (Junttila et al. 2009). Administration of this drug has some side effects concerning increased cardiotoxicity in older patients. To combat this concern of congestive heart failure associated with trastuzumab, the patients are administered with shorter durations of trastuzumab (Pivot et al. 2013). Another humanised monoclonal antibody in addition to trastuzumab, pertuzumab also prevents HER2 dimerisation by binding to its extracellular domain. In combination with trastuzumab, pertuzumab confers an antibody dependent cell mediated toxicity (Scheuer et al. 2009). Studies have shown that the combination of trastuzumab, docetaxel, and pertuzumab in metastatic breast cancer, have improved progression free survival rate of patients (Baselga et al. 2012). The use of aromatase inhibitors in addition to pertuzumab is a better combination for patients who can't receive chemotherapy. Ado trastuzumab emtansine known as TDM-1 is an antibody drug conjugate consisting of monoclonal antibody trastuzumab linked to a derivative of maytansine DM1, having cytotoxic effects (Phillips et al. 2008). Proteolytic degradation mediated cytosolic release of active DM1 due to receptor mediated endocytosis triggered by TDM-1 and HER2 receptor binding, causes micro tubule polymerisation and subsequent cell death (Poon et al. 2013). Compared to trastuzumab, TDM1 has lower incidence of cardiotoxicity in administered patients (Singh and Lichtman 2015).

Different class of drug used in breast cancer, lapatinib is a dual tyrosine kinase inhibitor that targets kinase domain of HER2 and is administered orally to patients (Bilancia et al. 2007). For patients having risk of congenital heart disease, lapatinib with endocrine therapy or capecitabine has been approved for administration, as trastuzumab enhances the risk of cardiotoxicity (Gradishar et al. 2018). In addition to this, lapatinib is also used in combination with other inhibitors to overcome the acquired resistance due to use of first line anti HER2 drugs. The adverse effects of using anti-HER2 drug are nausea, vomiting and diarrhea, but not cardiotoxicity. Another anti-HER2 drug, neratinib also inhibits HER2 kinase activity and downstream kinase pathways (Chan 2016). For patients in initial stages of HER2 tumors, post trastuzumab course are supplemented with oral administration of neratinib. Due to incidence of nausea and diarrhea, the patients are recommended to use loperamide with neratinib for initial two cycles. Currently various tumor vaccines consisting of tumor antigenic peptides, tumor cell lysates, DNA, immune adjuvants, etc. are being developed as an alternative for Mab.

Against HER2 positive breast cancer, NeuVax comprising of HER2 peptide E75 with adjuvant GM-CSF (granulocyte macrophage colony stimulating factor) is the developed vaccine that initiates HER2 specific $CD4^+$ and $CD8^+$ Th1 responses (Mittendorf et al. 2006). Neuvax is mainly restricted to HLA A2 and HLA A3

subtypes and also causes injection site erythema upon administration. These are also associated with multiple systemic toxicities which forced to stop administration of this vaccine.

15.5.3 Deubiquitinase Enzymes as Targets

The ubiquitin proteasome system labels the proteins targeted for degradation by an ubiquitin molecule and the 26S proteasome complex degrades these misfolded proteins into smaller peptides. Of the seven lysine residues in a ubiquitin molecule, chains of lys-48 tagged proteins are marked for degradation where lys-63 tagged proteins are degraded via non proteasomal processes such as the autophagy lysosome pathway (McKeon et al. 2015). Several members of USP family of deubiquitinases are involved with many different types of cancer (Pal and Donato 2014). Over expression of USP1, USP2, USP7, USP9X, USP10, and USP14 are associated with various malignant tumors. Increased levels of USP2a and USP7 was observed in biopsies of prostate cancer, which correlates with lower patient survival and poor prognosis (Baselga et al. 2012). The abnormal expression of the USP ubiquitinases are linked to its oncogenic properties that regulate its substrates Aurora A, MDM2, FASN. HBX19818 and its analogues HBX41108, P22077, P45204 have shown to inhibit the activity of USP7 and USP10 by interacting with the ES complex (Colland et al. 2009). USP7 inhibition by these inhibitors increases the NF-kβ ubiquitination thereby stabilising it and limiting the inflammatory response (Colleran et al. 2013). P22077 induces apoptosis and inhibits p53 mediated cell proliferation in neuroblastoma mouse models by inhibiting USP7 activity (Lim et al. 2016). Another USP inhibitor P5091 targets USP47 and USP7 in colon cancer cells (HCT 116) and multiple myeloma cells by stabilising p53 and reducing cancer cell growth (Pal and Donato 2014). Overexpression of USP14 is linked to the occurrence of breast cancer, multiple myeloma and lung adenocarcinoma (Zhu et al. 2016). USP14 inhibitor B-AP15 induces poly ubiquitination leading to oxidative stress and ultimately apoptosis. The development of anticancer small molecule inhibitors against USPs will open new treatment opportunities against fatal diseases.

15.5.4 Epigenetic/Chromatin Modulators as Targets

DNA methylation and epigenetic abnormalities associated with DNA damage are drivers of tumor initiation and progression. These changes in the chromatin are targets for therapeutic intervention in cancer to restore normal cellular epigenome (Fig. 15.1, Table 15.5).

The broad set of epigenetic reprogrammers includes inhibitors against DNA methyltransferases (DNMTi), histome deacetylases (HDACi) and bromodomain and extra terminal motifs (iBETs). The gene expression alterations due to cancer are reversed by the epigenetic inhibitors (Bhadury et al. 2014). DNMTi were initially developed for therapeutic purposes to reprogram the cancer epigenome. Azacitidine

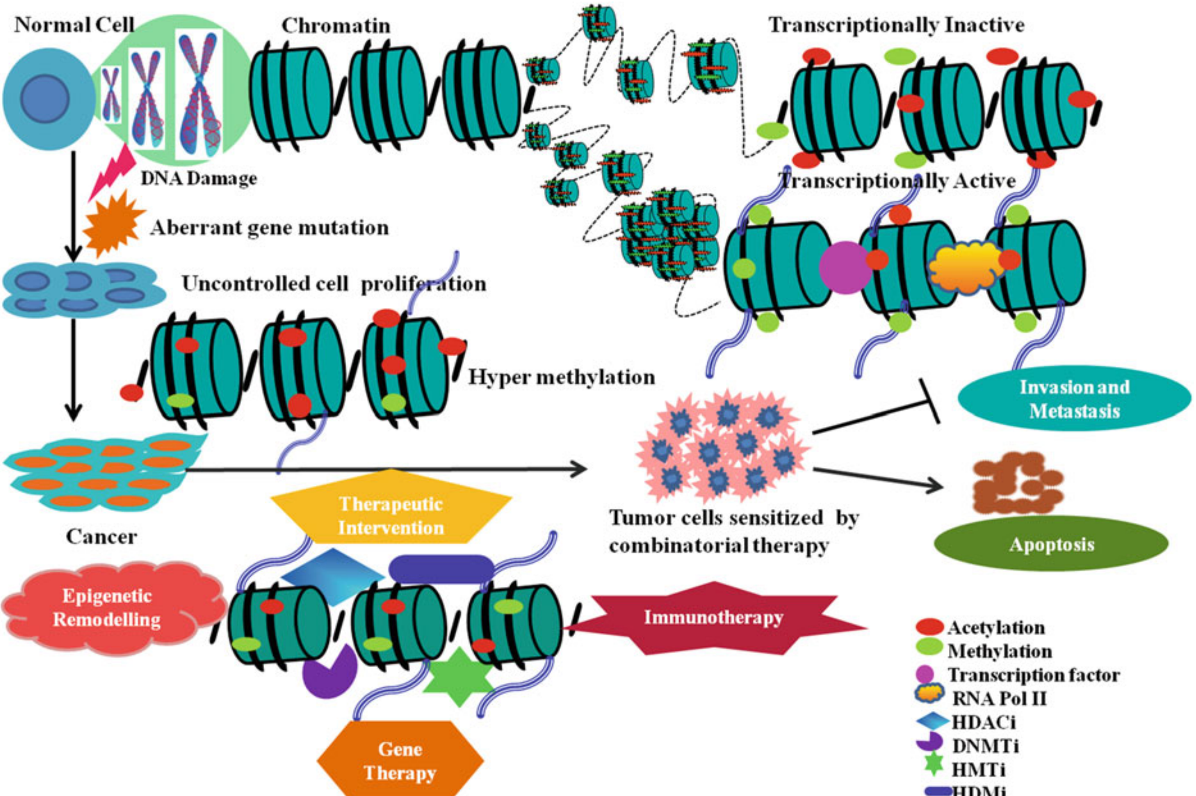

Fig. 15.1 Epigenetic reprogrammers in cancer therapy. For the treatment of peripheral T-cell lymphomas, HDAC inhibitors, vorinostat, romidepsin, and belinostat have been approved. Recently panobinostat, in combination with bortezomib, has been approved to treat drug resistant multiple myeloma patients

and its deoxy derivative decitabine at an optimum dose demonstrated efficient response and reduction of malignant cells in AML patients during clinical trials (Lübbert et al. 2016). This treatment also improved the survival rate and blood count of administered patients. Due to the positive responses in AML, the FDA approved use of DNMTi in cancer treatment. DNMTi can provide long term as well delayed individual responses depending on the epigenome reprogramming (Tsai et al. 2012). The major concern of (Tsai et al. 2012) therapy is its primary and secondary resistance and limited activity due to short half-life of these drugs (Stewart et al. 2009). To counteract these limitations, a second generation DNMTi, guadecitabine has been introduced which showed improved pharmacodynamics in clinical trials (Issa et al. 2015).

Another group of epigenetic therapy apart from the mentioned class of inhibitors are targeted therapy against specific genetic defects. Lymphoma mutations activate EZH2, a histone H3K27 methyl transferase and inhibitors against EZH2 induces killing of these cancerous cells (McCabe et al. 2012). There is aberrant hypermethylation due to mutation in IDH1 and IDH2 (TCA cycle genes) in acute myeloid leukemia (AML) and gliomas and clinical trials have shown that IDH inhibitors can be effective against AML (Litherland et al. 2015).

In addition to these, a combination of DNA methyl transferase inhibitors and histone deacetylase inhibitors can be a better therapeutic target and recent developments show enhanced efficacy of this combinatorial therapy. As methylated DNA regions are usually accompanied by reduced histone acetylation, the most common drug combinations are use of DNA methyl transferase inhibitors and histone deacetylase inhibitors. This treatment is initiated by providing low doses of DNA methyl transferase inhibitors followed by histone deacetylase inhibitors (Ahuja et al. 2016). The HDACi mostly act on the HDACs residing in nucleus, HDAC1 and HDAC2 (Falkenberg and Johnstone 2014). In preclinical studies this approach of using DNMTi and HDACi, increased antitumor responses and expression of silenced genes (Chai et al. 2008). At higher doses, several off-target effects of this approach have been observed and hence the potential of this treatment is being tested in smaller trials.

Many cancerous cells have evolved strategies to escape immune evasion, hence an approach with a combination DNMTi, HDACi and immunotherapy are being developed and used in clinical trials against non-small cell lung cancer (NSCLC). A combination of class I HDACi, entinostat and azacitidine at low doses followed by immune check point therapy had robust responses in NSCLC patients. The combination of epigenetic therapy along with immunotherapy, are currently being tested in multiple clinical trials.

Fig. 15.1 (continued) (Miguel et al. 2014). Bromodomain and extra terminal motif inhibitors target and reversibly bind to bromodomain of bromo domain containing protein 4 (BRD4), that encodes an acetylated histone mark reader responsible for over expression of myc oncogene (Mohammad et al. 2015). These classes of inhibitors are currently under clinical trials. Many DNMTi and HDACis used for various other cases are listed in Table 15.5

Table 15.5 Drugs targeting the epigenome

Inhibitor	Drugs	Disease	Target	Approval status
iBET	0TX015	Hematological malignancies	PAN BET	Phase I and II
	GSK525762	Solid tumors and hematological malignancies	PANBET	Phase I
	CPI-0610	Hematological malignancies	PANBET	Phase I
	BMS-986158	Solid tumors	PANBET	Phase I
	BAY1238097	Solid tumors and lymphomas	PANBET	Phase I and II
	FT-1101	AML and MDS	PANBET	Phase I
	TEN-010	AML, MDS, and solid tumors	PANBET	Phase I
DNMT	Decitatine	AML, MDS	Pan-DNMT	EMA and FDA approved
	Azacitine	MDS	Pan-DNMT	EMA and FDA approved
	Guadecitabine	AML	Pan-DNMT	Phase III
HDACi	Varinostat	Cutaneous T-cell lymphoma	HDAC Class I, II, III	FDA approved
	Belinostat	Peripheral T-cell lymphoma	HDAC Class I, II, IV	FDA approved
	Romidepsin	Cutaneous T-cell lymphoma	HDAC Class I	FDA approved
	Panbinostat	Multiple myeloma	HDAC Class I, II, IV	FDA approved
	CXD101	Solid tumors and hematological malignancies	Class I	Phase I
	ACY-241	Multiple myeloma	HDAC6	Phase I
	CUDC-907	Solid tumors and hematological malignancies	Class I and IIb	Phase I
	AR-42	Hematological malignancies	Class I, II, IV	Phase I

15.5.5 Cancer Cell Metabolism as Therapeutic Target

15.5.5.1 Targeting Catabolites, Anabolites, and Aminoacids

The heterogeneity of cancer cells is the probable reason behind increasing resistance to chemotherapy. Cancer cells go through both catabolic and anabolic pathways to maintain an invasive, continuous and progressive cell cycle. Tumor cells possess elevated metabolic activity due to higher rates of cell division and comparatively more consumption of nutrients than normal cells. The important metabolites utilised by tumor cells include glucose, pyruvate, hydroxybutyrate, acetate and fatty acids. Individual needs of tumor cells direct them toward uptake of different metabolites thereby channelling to different metabolic pathways. This scenario calls out for metabolic coupling. Tumor cells couple with each other in such a manner that, the metabolic by-product excreted by one tumor cell is used as substrate by the other. For example: In vitro model of cervical cancer and Colon cancer metabolise glucose to lactose which is then utilised by Normoxic cancer cells to form lactate for

mitochondrial oxidative phosphorylation (OXPHOS). The rate of production of ATP is more through Glycolysis-Lactate pathway but the yield is 36 times less as compared to TCA-OXPHOS pathway. Which pathway is to be followed by the tumor cell solely depends upon the individual metabolic needs. Higher metabolic rate of tumor cells paves us a way to develop anticancer therapies against these metabolites, but at the mean time understanding the intricate mechanisms of their metabolism is of extreme importance for determining the exact dynamics of pathway followed by the administered drug.

None of the existing anticancer drugs are profoundly established against tumor glycolysis. Although glucose-mediated pyruvate is used as a common substrate in intermediate reactions of TCA cycle, OXPHOS mediated glutamine breakdown is most predominantly used for ATP generation by cancer cells. Glutamine-derived end products like isocitrate and glutathione are used as a substrate for lipid synthesis and serve as antioxidant for tumor cells respectively. Glutaminase inhibitors like Bis-2-(5-phenyl acetamido-1,2,4-thiadiadol-2-yl ether sulfate) BPTES will cease any metabolic activity having glutamine as an intermediate. Glutamate is the precursor of a numerous amino acids like proline, alanine, and asparagine thus underlying the importance of its inhibitors. Lactate is predominantly generated in Fibroblast associated cancers. Lactate shuttle also persists in the non-cancerous tissues like monocytes, granulosa, and myocytes. Prevalence of this transporter in normal and cancerous cells will help to establish a comparative analysis on the effect of drugs targeting it. Anti-LDH (Lactate Dehydrogenase) drugs can be used for treatment of fibroblast cancers by targeting either transcription factors (or) Transport shuttles. The mitochondria-pyruvate shuttle helps in exchange of metabolic intermediates among different compartments within cancer cell. Developing therapies targeting pyruvate in its upstream and downstream nodes will help in understanding their efficacy in drug development.

The dependency of cancer cells on ketone bodies (β-hydroxy butyrate and oxaloacetate) through fatty acid oxidation increases during hypoxic and nutrient limiting conditions. Fatty Acid oxidation is one of the intermediate active processes that help in the maintenance of redox metastasis during nutrient deprived condition. In hypoxic and energy depleting conditions, cancer cells metabolise fatty acids by AMPK (5′AMP Activated Protein Kinase) to produce NADH and NADPH which substantially act as energy substitutes for their proliferation and survival. Drugs that target FAM (Fatty Acid Metabolism) can be used as an important therapeutic strategy. Recent studies have shown crucial role of amino acid and fatty acid metabolic pathways in cell proliferation which can also be attractive target for anticancer drugs in future. These studies highlight metabolic vulnerability of tumor cells which can further be used to exploit and design therapeutic drugs.

15.5.5.2 Targeting Processes Regulating Metabolism

Apart from being the ATP synthesising machinery, mitochondria is the potential hub for different catabolic and metabolic processes. There is a continuous efflux of proteins, small molecules like NADH, NADPH and carbon atom in and out of the mitochondrial compartment which take part in several signalling pathways.

Evidences suggest that de regulation of mitochondrial signalling pathways disrupts cell homeostasis and leads to tumor aggression. Highly invasive and proliferative tumors use OXPHOS as the energy source to carry out their metabolic activity (LeBleu et al. 2014). This is mediated through mitochondrial coupling to establish a feasible microenvironment. Therefore, study of mitochondrial biogenesis, maintenance of oxidative stress and redox potential can help us in designing potential targets for treatment of invasive tumors (Ferlay et al. 2015; Katajisto et al. 2015).

The alternate sources of energy for cancers like leiomyomas, pheochromocytomas, and paragangliomas are fumarate hydratase and succinate dehydrogenase. Mutation in these enzymes leads in reduced activity of OXPHOS which promotes cell survival. The exact mechanism as to how reduction in OXPHOS leads to increase in survival rates is still unclear. Probably the interconnection between mitochondrial biogenesis and oxidative stress might clear the reason behind this. Over utilisation of OXPHOS during mitochondrial biogenesis induces oxidative stress. Moderate oxidative stress and hypoxia promotes appreciable mitochondrial function whereas increased oxidative stress and hypoxia leads to its reduction. In depth understanding of oxidative stress vulnerabilities will help us in designing effective biomarkers that can be subjected to further clinical trials. Increase in ROS leads to cell death in normal cells whereas induction of oncogenic metabolites like MYC, KRAS and BRAS that act as antioxidants to protect cancer cells against ROS action. Thus, targeted therapy against oncogenic antioxidants can also serve as an intervention strategy. Table 15.6 mentions the potential inhibitors of cancer cell metabolism.

15.5.5.3 Targeting Transcription Factors

Transcription factors are important modulators in processing the individual oncogenes into substantial complexes so as to shape their potentiality of causing disease. For example: HIF-1 and MYC play a crucial role in reshaping the metabolic outline in progressive and proliferating cancer cells. Recent advancements suggest that there is a negative feedback loop of interaction between OXPHOS and HIF-1. HIF-1 induces gene expression of Pyruvate dehydrogenase kinase 1, PKM2 and Cytochrome oxidase 4-2 that downregulates OXPHOS mediated ATP generation. Inversely, metabolites generated by OXPHOS mediated signalling cascade, fumarate and succinate upregulates HIF-1. HIF-1 has two subunits; HIF-1α and HIF-1β. Both the subunits are validated to interact only under hypoxia conditions during which HIF1α expression is regulated by glycogen synthase kinase mediated by PI3/AKT signalling. The functionality of HIF-α as a oncogene (or) an immunosuppressant is still unclear. It is persuaded as an immune suppressant in metastatic and proliferative tumors but an oncogene in less malignant cancer cells. Further investigation is required to study the effect of HIF1-α inhibitors like Topotecan and Irinotecan on HIF1. According to literature, MYC-1 is the master transcriptional regulator which is deregulated in many cancers. Among the vast arena of MYC-1 regulated cells, only selectively active genes are up-regulated by MYC-1. It proposes conditional agonist and antagonistic interaction with HIF-1. It influences ribosomal regeneration, mitochondrial biogenesis, etc. MYC mutants display a very invasive,

Table 15.6 Cancer cell metabolites and their inhibitors

Cancer cell metabolite	Mechanism of action	Inhibitor
GLUT1 (Glucose transporter 1)	Transport of glycolytic products	Silibilin
PKM2 (Pyruvate kinase isoenzyme 2)	ATP production by pyruvate dephosphorylation	TLN-232
Glutaminase	Glutamine → Glutamate, Production of fatty acids, glutathione	CB-839
MCT (monocarboxylate transporter)	Lactate import and export from plasma membrane	AZD3965
Cas9 (Carbonic anhydrase 9)	Transcriptional target of Cas9	Indisulam
(LDHA) Lactate dehydrogenase A	Pyruvate	FX-11, galloflavin, GNE-140
(CPT1) Carnitine-O-palmitoyltransferase 1	Catalysis of rate limiting step in FAO (fatty acid oxidation)	Perhexilline, oxfenicine
(IDO) Indoleamine-2,3-oxygenase	Rate limiting enzyme in tryptophan metabolism	Epacadostat, indoximod
HMGCR (3-hydroxy-3-methyl glutaryl coenzyme A reductase)		Statin
NFR2 (Nuclear factor erythroid 2)	KRAS-induced cell proliferation and tumorigenesis	Bardoxolone methyl
HIF-1 (Hypoxia inducing factor 1)	Increases rate of glycolysis and lactate production	Digoxin
HSP90 (Heat shock protein)		Ganetesib

proliferative, and progressive version of cancer malignancy. INC054329 and CPI-0610 are the potential small molecule inhibitors that prevent the interaction between bromodomain and HIF-1 surface binding motif. Hence, a clear understanding about the mechanistic action of individual transcription factors in essential to get some insight into its connectivity with cancer progression.

15.5.6 Antibody Directed Enzyme Prodrug Therapy (ADEPT)

Most chemotherapeutic drugs for the treatment of cancer are quite nonspecific in action. To achieve improved specificity, new therapeutic strategies include the use of monoclonal antibodies targeted to specific tumor antigens for drug delivery options. The heterogeneous antigen expression of epithelial cancer cells and poor antibody-drug ratio limit the success rate of administered antibody-drug conjugates. The antibody drug conjugates after being internalised by the cells, release the drug intracellularly to exert its effect. ADEPT targeted therapy includes weakly toxic pro drug which when delivered to tumor target sites gets converted to an active cytotoxic drug (Denny 2004). Antibody directed enzyme pro drug therapy overcomes the limitations of internalisation and releases drug at the extracellular

regions of the tumor. Due to the effective progress of antibody drug conjugates, FDA has approved the application of Adcetris and Kadcyla for therapeutic purposes in adult patients with newly diagnosed Stage 3 or 4 classical Hodgkin lymphoma (cHL) and HER2 positive metastatic breast cancer respectively (Lambert and Chari 2014).

Monoclonal antibodies can deliver conjugated enzymes to tumor sites, where they can convert pro drugs to cytotoxic agents killing tumor cells. The drug released extracellularly, can diffuse into tumor cells killing both cells that express tumor antigen and those that do not express these antigens. Enzymes used for ADEPT therapy are classified into different classes, i.e., the enzymes of mammalian origin, enzymes of non-mammalian origin with a mammalian homologue, and enzymes of non-mammalian origin without a mammalian homologue. Many mammalian enzymes including human β-glucuronidase in combination with pro drug, forms a part of immunised antibody fragment containing fusion protein and is used as an ADEPT (Prijovich et al. 2016). Non mammalian enzymes such as β-lactamase and carboxypeptidase G2 have been reported for ADEPT therapy (Prijovich et al. 2016). As they elicit immunological reactions, these antibody drug conjugates have never been preceded to clinical trials. One of these antibody-drug conjugate system, has found potential clinical applications. The bacterial enzyme, carboxypeptidase G2 (CPG2) isolated from *Pseudomonas* sp., when conjugated to antibodies (non-internalising) targeting tumor associated antigens (hCG, human chorionic gonadotropin and CEA, carcino embryonic antigen) in combination with CMDA pro drug showed complete regression of chorio carcinoma in mice models. Many preclinical trials have supplemented the feasibility of antibody directed enzyme prodrug therapy, but the clinical data on ADEPT are quite limited and restricted.

15.6 Enzymes in Diagnostics

Accurate and in time diagnosis of a disease has become very crucial to reduce the mortality rate and ensure sound health. The tendency of pathogenic agents to continuously evolve depending upon their microenvironment and the limitations of existing diagnostic aids, have rationalised the need to develop more rapid and effective alternatives. The traditional principle of diagnostic kits, being antigen–antibody interaction is now being replaced by enzyme–substrate reaction. Enzymes in diagnosis can be explored as biosensors, biomarkers and reporters. They fulfil all the characteristics of a potential diagnostic agent i.e., sensitivity, specificity, reproducibility, robustness, and practicability of a reaction. The reliability and accuracy of enzyme driven diagnosis is improved when implemented in conjugation with apt molecular marker of a disease. For example: Quantiferron TB Gold in tube assay (QFT-GIT) in combination with adenosine deaminase is used for the detection of TPE (Tuberculosis Pleural Effusion) in tuberculosis (Zhang et al. 2016).

Enzymes are now being extensively used as “Point of Care” Diagnostics. Pyruvate Kinase is used as an efficient biomarker for colorectal cancer in Immunochromatographic Fecal M2-PK test kit. This kit also facilitates early stage detection. Enzyme-based diagnostic kits can be re-engineered to convert biological

signals into electrical interfaces. Personalised glucose meters are based on electrochemical reactions mediated by redox coupling with glucose strips. They lead to the activation of a cascade of enzymes which utilises the target. Until now PCR used to be the most sensitive method for detection of any mutation present but, emergence of combinatorial strategies have proven to be more promising against diseases like cancer and Malaria. Rolling Circle Enhanced Enzyme activity (RCA) with microfluidics Lab on Chip and Digital Droplet PCR are examples of such methods used for the detection of Topoisomerase I in malaria and Tyrosine kinases in Lung cell carcinoma respectively (Oskina et al. 2017). Also, there is development of a rapid and sensitive H5N1 HPAI virus specific diagnostic luminescence analyzer which contains one monoclonal antibody for trapping of immune complexes and other for chemiluminescence output by reducing the substrate (Tsunetsugu-Yokota et al. 2014).

Another example is the use of rapid sandwich immunoassay using fluorescent nanoparticle conjugated with Prostate Specific Antigen (PSA) antibodies in Prostate cancer (Park et al. 2014). Circulating cancer cells are targeted by enzymes before they change their confirmation according to the system suitability. Hormone suppressant therapy and HDAC inhibitors are combined in assay development to re-sensitise the unintentionally created ER-ve breast cancer cells against suitable inhibitors. This assay requires action of enzymes using ChIP-Seq followed by FACS. Application of enzymes as a reporter gene produced by virus during infection in detecting the antibodies against the target protein can tell us the efficacy of vaccine administered. A most common enzyme used as reporter is Alkaline Phosphatase. It helps in detecting the viral load in HPV infection. False diagnosis of a disease can mislead to lethal treatments with drastically delaminating after effects. Enzymes play a substantial role in identifying the major causative agent of infection. ELISA-based detection of antigens is till date sensitive assay to identify the symptoms that belong to *Blastomyces dermatidis*/*Cryptococcus neoformans*. Table 15.7 represents other applications of enzymes kit based diagnosis.

15.7 Emerging Enzyme Drug Delivery Platforms

Current research shows the application of enzymes in numerous maladies involving medial and clinical aspects. The enzyme therapies are less in use due to their potential off-target interactions. Recent studies show that these limitations can be circumvented by encapsulation (liposome mediated) and targeted delivery (peptide conjugation and nanocarriers).

The major advantages of bio conjugation and encapsulation for enzyme targeted therapy are:

1. its rapid uptake within the cells,
2. improved biotolerance,
3. enhanced biodistribution resulting in minimal off target toxicity, and

Table 15.7 Application of enzymes for diagnostic purposes

Disease	Principle
Glioblastoma	Quantitative measurement of MGMT (Methylguanine methyltransferase)
Diabetes	FPOX-mediated fructose-val-his catalysis to detect HbA1c level in blood
Breast cancer	Immunohistochemistry to detect HER2 expression
Hepatitis B	ELISA-based detection of HBsAg
HPV infection	In vitro neutralisation assay based detection of major capsid protein L1
Colorectal cancer	Digital droplet PCR to determine KRAS mutation
Viral gastroenteritidis	Biotin-Avidin based enzyme immunoassay
Aspergillosis	Fluorescent bead based immunoassay

4. protection of the targeted enzyme from degradation by host proteases and nucleases due to its prolonged life span upon encapsulation. Despite all these merits of encapsulation and bio conjugation, there are certain limitations such as eliciting an immune response by targeting ligands and proteolytic degradation of the enzymes leading to its inactivation.

Liposomes are vesicles that encapsulate many small molecules for targeted drug delivery. These drug delivery vehicles comprise of amphipathic lipid bilayers, and have recently emerged as therapeutic nano composites. The biocompatibility, stability, and biodegradable properties of liposomes make them better delivery platforms. For the treatment of lysosomal storage diseases (LSDs) new advances have been made and liposomes encapsulating β-galactosidase enzyme is used for the targeted delivery of specific compartments. This encapsulation protects β-galactosidase from proteosomal degradation and limits the risk of further immunological reaction. Liposomes containing cationic lipid formulations, such as dioleoyl phosphatidyl ethanolamine and trifluoroacetylated lipo polyamine (TFA-DODAPL:DOPE) have been demonstrated to deliver labelled antibodies, granzyme B, caspases, etc. inside the cells (Zelphati et al. 2001). Caspases are apoptotic mediators and upon delivery by cationic liposomes induce apoptosis. Another cationic formulation, bisguanidinium-tren-cholesterol:DOPE, has been used to deliver anti cytokeratin 8 antibody in case of cystic fibrosis (CF) and β-galactosidase for LSDs (Zuris et al. 2015). All these reports, help in establishing liposomes as a potential intracellular delivery platform.

15.7.1 RBC Carriers

One of the emerging forms of biological drug delivery systems are the RBC carriers. The major advantages of RBC carriers being used as a natural source of drug delivery system than the non-biological nano composites are as follows:

1. enhancement of pharmacokinetics.
2. has a potential not to invoke immunological responses.
3. the inner RBC volume is devoid of any cell organelles and this space can be utilised for drug encapsulation.
4. They exist in the body for about 3 months, which has a much longer lifespan than other nano composites.
5. Inexpensive, biodegradable, and biocompatible (Leuzzi et al. 2016)

Enzyme-based drugs can be fused inside the RBC via surface loading, membrane fusion, hypotonic loading, chemical conjugation, cell penetration, and genetic engineering. Studies have shown that β-glucuronidase encased inside the RBCs after being successfully delivered to mice deficient of β-glucuronidase, result in enhanced enzyme activity.

Surface loading of L-asparaginase and tissue plasminogen activator (tPA) have shown remarkable results in animal models. L-asparaginase used in the treatment of acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma was surface loaded on erythrocytes and showed 1000-fold reduction in immunogenicity and enhanced the pharmacodynamics by tenfold. Single administration of the erythrocyte conjugated L-asparaginase to mice improved their tolerance toward the doses of free enzyme (Lorentz et al. 2015). In another study, tissue plasminogen activator (tPA) surface loaded on erythrocytes, have shown ten times greater persistence in the circulation compared to the free enzyme (tPA). This resulted in enhanced fibrinolytic activity and better clot dissolution (Murciano et al. 2003). These studies demonstrate that erythrocyte mediated enzyme delivery is a safer course of treatment due to minimal immunogenicity. Previous surface loading of enzymes on RBCs was due to noncovalent binding, and recent reports have shown that the mouse RBCs were modified on their surface to present various functional moieties (Park et al. 2014). Here, they have modified the erythrocyte surface using enzyme Sortase, together with other recombinant proteins for targeted delivery by the enzyme.

There are multiple limitations of using RBC carriers, for example, in case of internal loading, there is damage to the RBC cell surface, leading to membrane leakage. In addition to this, there is premature removal of these enzyme carrying erythrocytes from the blood stream by macrophages. The major drawback faced is the challenge to engineer the RBCs for making it less immunogenic due to the difference in blood type matching of the loaded RBCs to that of the recipient.

15.7.2 Virosomes

Virosomes are nano particles made up of liposomes displaying viral proteins (neuraminidase and hemagglutinin) on their vesicular surface for drug delivery mechanism. Virosomes are considered very safe as they are devoid of any hazardous viral gene. Influenza virosomes are commercially available as vaccines against influenza (Felnerova et al. 2004). Other viruses such as Hepatitis B virus, Epstein-barr virus, Sendai virus, etc. act as scaffolds to develop virosomes.

In a study, virosomes based on Sendai virus, were engineered to display ScFvs (single chain variable fragments) on their surface for targeted delivery. This recombinant ScFv was used to deliver FITC (fluorescein isothiocyanate) conjugated lysozyme against an oncofetal antigen on HeLa cell surface. The active enzyme is delivered via ScFv mediated membrane fusion. Hence, modifying the liposome cell surface with specific ligands increases its binding to the cell surface. In addition to virosomes, certain viral like particles (VLPs) have been generated known as virus nano particles (Lee et al. 2016). The bacteriophage DNA packaging machinery, in a study, was used as a platform and the capsid was engineered to deliver proteins targeted to mammalian cells. The engineered capsid contained cell penetrating peptide on surface for targeted delivery. This system was used to successfully deliver β-galactosidase to cells deficient of this enzyme. The T4 bacteriophage capsid as a therapeutic delivery vehicle is better due to reduced toxicity and inability to infect mammalian host cells (Tao et al. 2013). Similarly in other reports, it was shown that alcohol dehydrogenase D was encapsulated inside the capsid of P22 bacteriophage. The cytochrome P450 was encapsulated inside P22 bacteriophage capsid and was delivered to cervical cancer cells, where the enzyme was functionally active (Sánchez-Sánchez et al. 2015). These studies demonstrate the use of virosomes, viral nano particles and bacteriophage capsid NCs as new age delivery systems for therapeutic purposes.

15.8 Conclusion

Enzymes play central role in healthcare. Initially various enzymes have been identified as therapeutic targets and several drugs have been developed. Several enzymes are used in drug synthesis and disease diagnostics. Advent of molecular cell biology and immunology has led to the identification of several new pathways which are extensively explored for development of new therapeutic interventions. Over the last two decades, many enzymes are developed as direct therapeutic agents due to their high specificity and less side effects. Recent advancements like production of humanised enzymes, encapsulated enzyme therapy, antibody-mediated drug delivery, enzyme replacement therapy, etc. are actively augmenting traditional chemically synthesised drugs and even have potential to replace them altogether in some cases. New insights in fundamental biology and disease biology, along with knowledge about successful drugs and their targets will pave the way for discovery of efficient new generation therapeutics.

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16 L-Asparaginase and Methioninase as Prospective Anticancer Enzymes: Current Applications and Production Approaches

Smruti Malinee Sahoo and Sabuj Sahoo

Abstract

Since the first documented case of cancer in Egypt in 1500 BC till now, cancer accounts second position in death rate in the world. International Agency for Research on Cancer and WHO reported 18.1 million new cases and 9.6 million deaths due to cancer during 2018 worldwide. In India, 9.81% of male and 9.42% of female are at a risk of developing cancer before the age of 75. Chemotherapy, radiation therapy, immunotherapy are the treatments for cancer but have side effects like hypersensivity, pancreatitis, thrombosis, coagulopathy, hyperglycemia, etc. L-asparaginase and methioninase are the widely used protease enzymes for cancer treatment. Cancer cells depends on external source of amino acids for synthesis of protein for their growth, administering these protease enzymes in the body will cleave the excess amino acid compelling the cancer cell starve to death. Native form of these enzymes shows several side effects like allergic reaction in patients. To eliminate such side effects genetic engineering has been employed to construct recombinant drugs (e.g. Elspar, Oncospar) with high specific activity, half-life and less toxic effect. With optimised fermentation techniques the recombinant drugs can be scaled up to satisfy the market demand. This review emphasises on the eminent sources, mechanism of action, purification, recombinant techniques and optimised fermentation methods used for production of L-asparaginase and methioninase.

Sabuj Sahoo was deceased at the time of publication.

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Keywords

Recombinant · Fermentation · Purification · Anticancer

16.1 Introduction

Second position of cancer in causing death has raised urgency for new therapies for cancer treatment. Both male and female below the age of 75 are at high risk of cancer. Indian Institute of cancer prevention and research reported oral cavity cancer tolls over 25% of cancer death in males however breast cancer and oral cancer accounts for 25% cancer in females. WHO reports approximately 3,000,000 children cancer cases between age group of 0–9 each year. Eighty percent of the child cancer is treated completely in high income countries while only 20% of the cancer patients get complete treatment in low and middle income countries (Steliarova-Foucher et al. 2017). Cancer treatments like chemotherapy, radiation therapy and immunotherapy are costly with side effects (Roy and Saikia 2016). Anticancer enzymes which cleave specific amino acids required by cancer cells are used in huge amount thereby leading a hope in cancer therapeutics. Amino acids are responsible for maintenance of normal function of cell with specific role in different biosynthesis pathway while any deviation in pathway due to insufficiency of amino acid may tend to cell death. Anticancer enzymes break down the amino acids and make the cancer cells thirst to death (Ananieva 2015). Normal cells have its own mechanism to regress the by-product formed and utilise it for its sustenance. L-arginase, L-asparaginase, L-glutaminase and L-methioninase are routinely used anticancer enzymes in treatment (Prajapati and Supriya 2017). These enzymes are associated with some side effects like hypersensitivity, hypoglycemia, nausea thus restricts its application in native form. Application of recombinant engineering in manufacturing of the drugs can exterminate the negative after effect of drug (Gurung et al. 2013). Genetic engineered drug will have better longevity inside cell with desirable specificity to act. Furthermore high demand of genetically engineered drug can only be contented with application of appropriate fermentation technique applied. This updated review accounts on L-asparaginase and L-methioninase as potential anticancer enzymes sources, assay methods, recombination, purification and suitable fermentation techniques.

16.2 Anticancer Enzymes

The main concept behind anticancer enzymes is cleaving particular amino acid and making it unfit for further utilisation by cells. These enzymes are target specific and alter substrate to a desired form. This type of conversion only affects the cancer cells as they are deficient in armamentarium required for function of cells whereas normal cells remain unaffected since they can revert those by-products formed by hydrolisation and utilise them. L-arginase (E.C.3.5.3.1), L-asparaginase

(E.C.3.5.1.1), L-glutaminase (E.C.3.5.1.2) and L-methioninase (E.C.4.4.1.11) are some of the anticancer enzymes in recent focus (Fernandes et al. 2017). L-asparaginase breaks L-asparagine lead factor for RNA and protein synthesis into L-aspartic acid and ammonia. Since normal cells can convert aspartic acid into asparagines through asparagine synthetase present on chromosome number 7 (7q21.3) so they are not affected. L-asparaginase is used in treatment of chronic lymphocytic leukaemia, lymphosarcoma, acute myelocytic leukaemia and melano sarcomas, Hodgkin's disease and also restricts formation of acrylamide in food (Kiriyama et al. 1989). L-glutaminase apart from treating acute lymphoblastic leukaemia it also used for treatment of irritability, anxiety, insomnia and to prove the quality of soy sauce. L-glutaminase cleaves L-glutamine essential for protein turnover, synthesis of nucleotides, immunity and hexosamines, energy production, redox homeostasis to glutamic acid and ammonia. L-glutamine is hugely required by cancer cells as oncogenic expression of C-Myc gene present on chromosome 8 codes for the transcription factor promoting expression of glutamine transporters (El-Asmar et al. 1965). L-methioninase used for treatment of wide range of cancer of breast, colon, kidney, lung cancer and brain and also act registered its use as antibacterial, antiprotozoal, antifungal and antioxidant agent (Breillout et al. 1990). Shortage of methionine in blood stream arrests the carcinoma cells at S-G2 phase of cell cycle resulting in cell death (Hoffman 1984). Apart from being an anticancer enzyme arginase is used for conservation of semen stock, treatment of Hepatitis-B, osteosarcoma and rheumatoid arthritis therapy. The nucleophilic attack of water molecule with Mg^{+} helps arginase to cleave L-arginine to L-ornithine and urea making cancerous cell starve to death (Prajapati and Supriya 2017).

16.3 L-Asparaginase

L-asparaginase (L-asparagine amido hydrolase E.C.3.5.1.1) is been used to treat lymphocytic leukaemia, chronic lymphocytic leukaemia, lymphosarcoma, acute myelocytic leukaemia, melano sarcomas and Hodgkin's disease, as L-asparagine is hydrolised to L-aspartic acid and ammonia (Meghavarnam and Janakiraman 2015). Apart from being an oncolytic enzyme it also helps in acrylamide reduction which is formed at the time of baking or cooking at high temperature due to reaction of asparagine and sugar (Vidya et al. 2011). L-asparaginase (L-ASPnase) has been categorised into two types: Type I and Type II. L-ASPnase Type I (ansA) found in cytoplasm is reported to have slow affinity enzyme activity while Type II L-ASPnase found in periplasmic space has high affinity enzyme activity. Type II comes into play in anaerobic state and exhibits significant antitumour activity (Batool et al. 2016). Mostly all L-ASPnase are identified to have tetramer structure of similar subunits namely A–D and their molecular mass ranges from 140 to 150 kDa. Each monomer has 330 amino acid residues which constitute 14 β-strands and eight α-helices. These are sorted into two recognisable domains i.e. a bulky N-terminal domain and a shorter C-terminal domain linked by 20 residues (approx.) (Pourhossein and Korbekandi 2014). The active site of L-ASPnase enzyme consists of conserved

residues. Ductile loop in active site consist two key residues Threonine-12 and Tyrosine-25. Out of these two residues, Threonine-12 is responsible for acylation reaction (Aghaiypour et al. 2001).

16.3.1 Mechanism of Action

L-asparagine being nonessential amino acid is required by cancer cells externally to synthesise protein for its function. The normal cells have L-asparagine synthetase which converts aspartic acid to L-asparagine for further protein synthesis but the cancer cells are deficient of asparagine synthetase as result cell depends on external source of L-asparagine from the blood. To treat cancer when L-ASPnase is induced in the body it hydrolysis the exogenous L-asparagine to L-aspartic acid and ammonia (Fig. 16.1). L-ASPnase shortage results in death of the cells as it cannot synthesis protein (Müller and Boos 1998).

16.3.2 Sources of L-Asparaginase

L-ASPnase produced by extended list of organisms like bacteria, yeast, fungi, algae and actinomycetes. L-ASPnase from *Erwinia carotovora* and *Escherichia coli* are preliminary source registered huge medical interest for treatment of leukaemia. *Pseudomonas florescens* (Mardashev et al. 1975) and *Mycobacterium phlei* (Pastuszak and Szymona 1976) has been reported to be good sources of L-ASPnase. *Thermus aquaticus* (Curran et al. 1985) stable at high temperature (60–70 °C) showed no activity with glutamine and D-asparagine is a potential source of L-

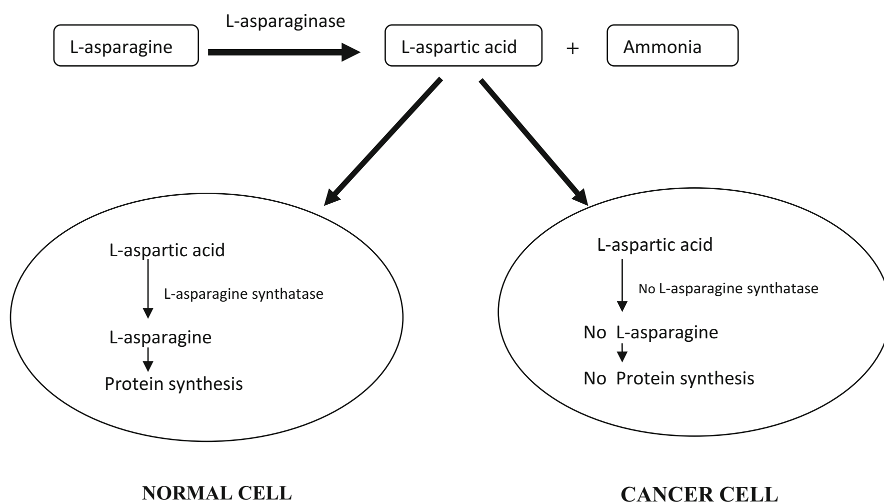

Fig. 16.1 Mechanism of action of L-asparaginase

ASPnase at industrial level. Pritsa and Kyriakidis in 2001 reported *Themus thermophilus* does not hydrolyze L-glutamine thus makes it suitable enzyme for clinical case study. L-ASPnase from yeast is detected to be less injurious to health. Dunlop and Roon reported *Saccharomyces cerevisiae* as source for L-ASPnase in 1975. Foda et al. (1980) reported *Rhodosporidium* sp., and Ramakrishnan and Joseph reported *Rhodosporidium toruloides* in 1996 as good source for L-ASPnase. Nagarajan et al. (2014) reported an endophytic fungus Alternaria sp. isolated from the leaf of *Withania somnifera* of Western Ghat hills showing glutaminase free asparaginase activity. *Aspergillus niger* recorded to produce high levels of L-ASPnase when grown on wastes of leguminous plants (Mishra 2006). Hendriksen et al. (2009) reported *Aspergillus oryzae* for L-ASPnase production and its use in food industry. Baskar and Renganathan (2009) reported *Aspergillus terreus* MTCC 1782 which produces good quantity of L-ASPnase with use of natural substrate like groundnut oil cake and corn flour in addition to media. Dhevendaran and Anithakumari in 2002 have reported another *Streptomyces* sp. isolated from fish *Therampon jarbua* and *Villorita cyprinoides* with L-ASPnase activity. *Chlamydomonas* sp. is reported to be first marine microalgae from which L-ASPnase has been extracted. *Vaucheria uncinata* is another yellow green algae to produce L-ASPnase (Batool et al. 2016; El-Naggar et al. 2014).

16.3.3 L-Asparaginase Assay

Enzyme action can be assayed through calculation of total ammonia released in presence of the substrate. In plate assay (qualitative) method the microorganism growth medium i.e. M9 medium or czapek dox media is supplemented with substrate L-asparagine and a pH indicator phenol red. L-ASPnase hydrolyses L-asparagine to aspartic acid and ammonia which is detected in form of colour change from yellow to pink as pH of the medium changes. Substrate–enzyme reaction is also detected by spectrophotometry (quantitative) with the use of Nessler's reagent at 450 nm (Imada et al. 1973). Another quick method of L-ASPnase activity study is through measuring ammonia through ammonia electrode.

16.3.4 Fermentative Production of L-Asparaginase

Efficient production of L-ASPnase has been reported through solid state fermentation (SSF) and submerged fermentation (SMF) techniques. Different carbon and nitrogen source, variation in pH, temperature, agitation and aeration has been applied for high yield of L-ASPnase production. Submerged fermentation involves growth of microorganism in liquid media at a high concentration of oxygen and moisture content whereas solid state fermentation system uses solid substrate such as agricultural waste is used for production. It utilises a little water as a result there is less chance of contamination. Product yield is more in case of SSF than SMF (Doriya et al. 2016; Cachumba et al. 2016). The substrate used and parameters of fermentation varies for various microorganisms and has been listed in Table 16.1.

Table 16.1 Fermentation details for production of L-asparaginase

S. No.	Microorganisms	Fermentation type	Substrate used	Parameters	Specific activity
1.	*Pseudomonas aeruginosa* 50071	SSF	Soy bean meal	Temp. 37 °C; pH 7.4; moisture content 50% (w/v)	142.18 IU/g
2.	*B. circulans* (MTCC 8574)	SSF	Red gram husk Ground nut cake Coconut oil cake	Temp. 30 °C; pH 7.0; moisture content 40% (w/v)	780 U/g of dry substrate 360 U/g of dry substrate 380 U/g of dry substrate
3.	*Aspergillus terreus*	SMF	Proline medium 2	Temp. 30 °C, pH 6.2	58.8 U/L
4.	*Erwinia aroideae*	SMF	Tryptone, glucose, yeast	Temp. 28 °C, pH 7	1250 IU/g dry weight of cells
5.	*Bacillus* sp.	SMF	Glucose	Temp. 37 °C, pH 5	157.03 IU/mL
6.	*Thermus thermophilus*	SMF	Tryptone, yeast extract, NaCl, glucose, $FeCl_3$, $MgCl_2$; $CaCl_2$	Temp. 70 °C, pH 7.0	–
7.	*Escherichia coli*	SMF	Terrific broth	Temp. 37 °C, pH 7.2	–
8.	*Aspergillus niger*	SSF	Glycine max	Temp. 30 °C; pH 6.5; moisture content 70% (w/v)	40.9–3.35 U/g of dry substrate
9.	*Streptomyces* sp. TA22	SMF	Sucrose	Temp. 27 °C, pH 7	390 IU/mg
10.	*Cladosporium* sp.	SSF	Wheat bran	Temp. 30 °C, pH 5.8	5.86 U/g of dry substrate

16.3.5 Purification

Different purification techniques have been used to purify L-ASPnase to have enzyme of good quantity and efficient activity. Ammonium sulphate precipitation, ion exchange gel filtration, alkaline lysis, dialysis and crystallisation are some the process employed for purification. As bacterial enzymes are quite acidic in nature cation exchange chromatography is generally used. To reduce the glutaminase

presence in L-ASPnase, the enzyme is purified by ammonium sulphate precipitation preceded by DEAE column chromatography and Sephadex G-100 chromatography. In case of recombinant enzyme the purification is done by Ni-NTA chromatography. Mostly, the purification steps are carried out at lower temperature and pH range of 7–8. In case of thermo stable organisms the purifications can be done above room temperature (Kumar et al. 2011).

16.3.6 Production of Recombinant L-Asparaginase

Short half-life and its high immunogenicity makes native form of L-ASPnase unfit to be used for medical purposes. With the use of recombinant DNA technology this oncolytic enzyme can be modified and can be used efficiently in therapeutics. The gene ans A or ans B is amplified, cloned and expressed in a suitable host system. The host system can be bacterial host system such as *E. coli* BL21 (DE3) or yeast host system like *Pichia pastoris*. The microbial genes, host cell and plasmid used for production of Recombinant L-ASPnase (Zuo et al. 2015) are listed under Table 16.2.

16.3.7 Commercially Available Drugs

Elspar is available in the market in form of white lyophilized plug or powder, each vial contains 10,000 IU of asparaginase and 80 mg of mannitol. It is recommended to take 6000 IU/m^2 intramuscularly (IM) or intravenously (IV) three times a week.

Table 16.2 List of recombinant L-asparaginase

S. No.	Source for the gene	Host cell	Plasmid used for recombination
1.	*Erwinia carotovora* (NCYC 1526)	BL21(DE3)pLysS *E. coli*	T7 expression vector
2.	*E. coli* MTCC 739	*E. coli* BL21(DE3)	pET-20b vector
3.	*Yersinia pseudotuberculosis* IP 32953 strain	*E. coli* BL21(DE3), JM 83, and TOP 10	pET23a and pBad24 vector
4.	*Enterobacteriaceae*	*E. coli* BL21(DE3)pLysS	pET20b
5.	*E. coli* MTCC 739	pPink host (*Pichia pastoris*)	plasmid pPink α-HC
6.	*Pseudomonas fluorescens*	*E. coli* BLR(DE3)	pET101
7.	*Synechococcus elongatus*	BL21(DE3)	plasmid pET22b (+)
8.	Nocardiopsis alba NIOT-VKMA08	Cloning vector pTZ57R/T and expression vector pQE30	*E. coli* JM109, *E. coli* M13
9.	*Mesoflavibacter zeaxanthinifaciens*	pET-16b vector	*E. coli* BLR(DE3)
10.	*Halomonas elongata*	pET21a vector	BL21(DE3)

Anaphylaxis, serious thrombosis, coagulopathy and posterior reversible encephalopathy syndrome, hyperglycemia are some of the side effects of Elspar. It can be taken by pregnant women in special cases. Oncaspar® (pegaspargase) is L-ASPnase that is covalently conjugated to monomethoxypolyethylene glycol (mPEG). Approximately 69–82 molecules of mPEG are linked to L-ASPnase; the molecular weight of each mPEG molecule is about 5 kDa. 2500 IU/m^2 of Oncaspar® is recommended intramuscularly or intravenously. It is advised to administer Oncaspar® at 14 days interval. Oncaspar® may show some allergic reaction such as hives, difficulty in breathing, swelling of face, lips and tongues in some patients. Due to possible risk to the infants, intake of the drug while breast feeding is not recommended. ERWINAZE (asparaginase *Erwinia chrysanthemi*) contains an asparagine specific enzyme derived from *Erwinia chrysanthemi*. ERWINAZE is available as a sterile, lyophilized, white powder in vials containing 10,000 IU of L-ASPnase each with sodium chloride (0.5 mg), glucose monohydrate (5.0 mg). 25,000 IU/m^2 of pegaspargase is recommended for administration intramuscularly or intravenously thrice a week. Grade 3 and 4 hypersensitivity has been reported in 5% of the patients with intake of the drug. Four percent pancreatitis and 5% glucose intolerance cases have been reported as side effect of ERWINASE.

16.4 Methioninase

L-methioninase (EC.4.4.1.11) also known as methionine-gamma-lyase, methionine demethylase, methionine lyase is used against wide range of tumour cell lines like glioblastoma, lung, kidney, colon and breast cancer. L-methioninase (L-METnase) is a pyridoxal-L-phosphate (PLP) dependent enzyme which catalyzes α, γ-elimination of L-methionine to α-ketobutyrate, methanethiol, and ammonia (Ruiz-Herrera and Starkey 1969). Methanethiol, deamination product of L-methioninase reacts with acyl-coenzyme A to release sulphur containing compounds such as dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS) and S-methylthioesters which plays an important role in flavouring of cheese and for its specific aroma (Yvon et al. 1997). L-METnase exists in tetramer form at liquid state and each monomer folds into three different domains having specific function. First domain includes 1–63 residues forming an N-terminal consisting of two helices and three β-strands. 1–39 residues stabilises the interactions of dimmers whereas 34–63 residues constitutes the active sites. The second domain is of 64–262 residues having eight α-helices with seven β-sheets inside it. It is a bulky PLP binding domain. The residues from 263 to 398 are the third domain forming C-terminal consisting of five β-sheets with five α-helices on either side of it (Sridhar et al. 2000). The active dimer is stabilised by formation of tetramer and quaternary association to one another. The open reading frame for the enzyme isolated from various sources ranges from 1170 to 1179 base pairs. The gene weighs about 42–43 kDa per subunit.

16.4.1 Mechanism of Action

The hydrolysis activity of L-METnase to α-ketobutyrate, methanethiol and ammonia is quite complex consecutive series of reaction. L-methionine has role in DNA methylation, polyamine synthesis, methylation reactions, cytoprotection, mammalian protein synthesis, antioxidative stress defense, synthesis of vitamins and antioxidants controlled gene expression. In presence of L-METnase the cancer cells starve for methionine required for metabolism of macromolecules and results in cell death. The Mechanism of L-METnase hydrolytic activity (Tanaka et al. 1985; Chin and Lindsay 1994; Faleev et al. 1996) is depicted in Fig. 16.2.

16.4.2 Sources of L-Methioninase

L-METnase is found in wide range of organisms such as bacteria, fungi actinomycetes, protozoa and plants. The gram negative bacteria are more searched than gram positive bacteria for L-METnase production. Some of the potential bacterial sources for L-METnase are *Achromobacter starkey*, *Idiomarina* sps., *Bacillus subtilis*, *Brevibacterium linens*, *Citrobacter freundii*, *Clostridium sporogenes*, *Micromonospora echinospora*, *Treponema denticola*, *Fusobacterium nucleatum*, *Pseudomonas putida*. Fungal source for L-METnase shows less immunogenicity

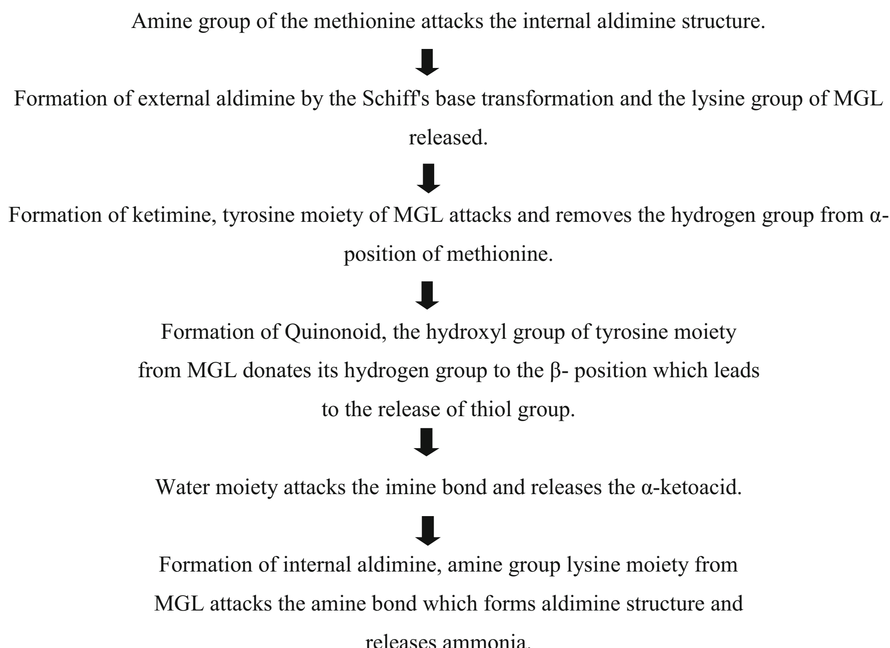

Fig. 16.2 Mechanism of L-methioninase hydrolytic activity

during therapy and its production through fermentation is cost effective. *Aspergillus niger*, *Aspergillus flavipes*, *Clonostachys rosea*, *Fusarium nivale*, *Candida trophicalis*, *Saccharomyces cerevisiae*, *Cladosporium oxysporum* are some the fungal sources for L-METnase production. Anaerobic parasitic protist *Entamoeba histolytica* and *Trichomonas vaginalis* produces isozymes of METnase i.e. MGL1 and MGL2. *Arabidopsis thaliana*, *Catharanthus roseus*, *Solanum tuberosum*, *Cucumis melo* are some of the plant source for L-METnase (Suganya et al. 2017).

16.4.3 L-Methioninase Assay

Qualitative analysis of the enzyme is done by growing the source microorganism on specific media supplemented with substrate methionine and phenol red. The change in colour of the plate to pink due to ammonia release depicts L-METnase production by the microorganism. Under standard conditions 1 IU L-METnase is defined as the amount of enzyme required to release 1 μM of methanethiol per minute incubated at 50 °C for 40 min. The released methanethiol reacts with Ellman's reagent (DTNB) (5,5′-dithio-bis-2-nitrobenzoic acid) to give a coloured compound TNB (2-nitro-5-thiobenzoic acid) which can be measured in visible range of spectrophotometer. The other product α-ketobutyrate is made to react with MBTH (3-methyl-2-benzothiazoline hydrazone) to give 2,4-dinitrophenylhydrazone which is then measured in TLC (thin layer chromatography) in terms of Rf values. Ammonia released in the hydrolysis process can be measure with the use of Nessler's reagents at 450 nm (Takakura et al. 2004; Johnston et al. 1981).

16.4.4 Production of L-Methioninase

Solid state fermentation (SSF) and sub merged fermentation (SMF) are two types of fermentation techniques used to fulfil the demand of L-METnase for cancer therapy. In case of submerged fermentation the methionine present in the aqueous medium gets rapidly oxidised in presence of reducing sugar and ions via Maillard reaction to form amadori compounds. Due to this Maillard reaction the availability of carbon and nitrogen source for microorganism reduces and makes it difficult for the microorganism to grow on it. Solid state fermentation uses agro-waste (Suganya et al. 2017) as substrate and reduces the contamination chances as moisture content is very less. The fermentative production of L-methioninase enzyme is given in Table 16.3.

16.4.5 Purification

To increase the enzyme activity different purification techniques are used such as dialysis, ion exchange chromatography, ammonium sulphate precipitation. Buffer used for purification contains 20 μM Pyridoxal 5′-phosphate to conserve the activity

Table 16.3 Fermentation details of L-methioninase enzyme (Suganya et al. 2017)

S. No.	Microorganism	Fermentation type	Substrate Used	Parameters
1.	*Arabidopsis thaliana*	SMF	Luria broth	Temp. 30 °C, pH 8
2.	*Cucumis melo (melon)*	SMF	Luria broth	Temp. 30 °C, pH 8
3.	*Aspergillus flavipes*	SSF	Wheat bran, wheat bran, wheat flour, cotton seed, lentil hulls, soya bean, feather chicken	Temp. 37 °C, pH 7
4.	*Pseudomonas ovalis*	SSF	L-methionine, urea, glycerol, KH_2PO_4, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, yeast extract	Temp. 37 °C, pH 7.2
5.	*Pseudomonas putida*	SMF	Glycerol, methionine medium prepared in PP buffer	Temp. 37 °C, pH 8

of enzyme (Kreis and Hession 1973). The crude enzyme is passed through DEAE-cellulose anionic exchange chromatography followed by Sephadex G-200 chromatography. The flow-through is collected for enzyme activity and protein quantification. The aliquot is also passed through SDS-PAGE electrophoresis to check the size of the protein. The recombinant enzyme is associated with 6×-his tag sequence so it is purified through Ni-NTA chromatography technique.

16.4.6 Recombinant L-Methioninase

Cancer therapy with L-METnase administration shows some gastrointestinal reactions such as reduced appetite, nausea and weight loss. This kind of hypersensitivity and increasing demand of L-methioninase for therapeutics use can be achieved through recombinant technology. The targeted gene is isolated, modified, cloned and expressed in a suitable host system. *Arabidopsis thaliana* is cloned into pET-43 and *Cucumis melo* is inserted into pET21a vector then expressed in *E. coli* host cell (Goyer et al. 2007; Gonda et al. 2013). *Entamoeba histolytica* is cloned into pGEX-6P-1 vector and expressed as a fusion protein with Glutathione S-transferase (GST) is purified using GSTrap HP column (Sato et al. 2006). *E. coli* BL21 and *E. coli* JM109 are the most used host system for expression of recombinant enzyme.

16.5 Conclusion

Increased death rate of cancer patients in low and middle income countries demands low cost drug with good specificity and high activity. Usage of classic cancer treatments resulted in various side effects with low activity. Genetically engineered

anticancer enzymes (L-asparaginase and L-methioninase) have capability to mitigate present demand of drug with less hypersensitivity. Choosing cheaper source of enzyme isolation, purification methods and suitable fermentation methods are preferred to lower the cost of the drug further. Hence, this review summaries some common sources, isolation techniques, assay methods, recombinant techniques and fermentation techniques for successful treatment of cancer.

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Production of Thrombolytic and Fibrinolytic Proteases: Current Advances and Future Prospective

17

Susmita Ghosh, Saheli Saha, and Sabuj Sahoo

Abstract

Cardiovascular diseases are one of the leading causes of death, globally. Thrombolytic and fibrinolytic therapies are effective treatments to dissolve the blood clots, improve the blood flow rate, and thereby, prevent tissue and organ damage. The advent of thrombolytic therapy, facilitated in easing the huge burden of cardiovascular diseases on the medical practitioners and provided them with a potential treatment for diseases like pulmonary embolism, myocardial infarction and deep vein thrombosis. Among all the thrombolytics, protease like Streptokinase, Urokinase, Nattokinase, and Tissue plasminogen activator (tPA) holds the majority. Thrombolytics are used as curative agents rather than prophylactics, administered to the site of blockage intravenously, or via long catheter or as dietary supplements. Despite the huge demand for thrombolytics, several factors like strain instability, cost-effectiveness, and lack of infrastructure contribute to the insufficient supply by the production units. Since, conventional production methods are inadequate to meet the spiking demands, high-throughput techniques like genetically engineered strains and fermentative methods are currently being preferred. Here, we discuss some present high-throughput production techniques, and also give an insight into potential ways of improving the yield, not only by adapting to high-throughput technologies but also by considering the eco-evolutionary history of the production strains.

Sabuj Sahoo was deceased at the time of publication.

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Keywords

Proteases · Thrombolytic · Fibrinolytic · Therapeutics · Genetic engineering · Fermentation

17.1 Introduction

Thrombolytic therapy or fibrinolytic therapy is used as an effective treatment to dissolve the blood clots in blood vessels, improve the blood flow rate and finally to prevent the tissue and organ damage. These thrombolytic agents either act as plasminogen activator which convert plasminogen to active plasmin or lyse the thrombi in the occluded blood vessel, thus dissolute the blood clot. Thrombolytics are used as curative agents rather than prophylactics. These agents can be administered to the site of blockage by intravenous injection or via long catheter; in some places like Japan it is used as dietary supplement.

WHO reports cited the death of about 17.9 million people each year due to Cardio Vascular Diseases (CVD) (Kaptoge et al. 2019) and an estimated 31% of all deaths worldwide among which approx. 75% of CVD deaths occurred in low-income and middle-income countries. Eighty-five percent of all CVD deaths are majorly due to heart attacks and strokes. Among all these diseases, myocardial infarction holds the majority followed by pulmonary embolism caused by the blockage of blood flow rate by fibrin clots. Although a large number of population is benefitted from the thrombolytic therapy, the prehospital delay, cost-effectiveness, and lack of infrastructure are the main barriers of this therapy even in developing countries, hence increasing the necessity of large production of all these agents in a cost-effective manner.

Among all the thrombolytics, protease holds the majority. Mostly used proteases are Streptokinase, Urokinase, Nattokinase, and Tissue plasminogen activator (tPA) (Chandramohan et al. 2019). Proteases registered its importance in regulation of major biological and pathophysiological processes viz., homeostasis, blood coagulation, fibrinolysis, and tumor progression attribute to their implications as agents meant for therapeutic interventions. The diagrammatic representation of fibrinolytic cascade is shown in Fig. 17.1. There are a number of compounds which inhibit fibrinolysis either acting on plasminogen and plasmin directly or inhibiting the enzymes involved in fibrinolytic method. For example, aminocaproic acid which binds to the kringle domain of plasminogen to inhibit fibrinolysis (https://www.drugbank.ca/drugs/DB00513) whereas alpha2-antiplasmin inhibits by forming a complex with plasmin (Reed et al. 2017). On the other hand, plasminogen activator inhibitors impede the fibrinolysis indirectly by inhibiting the plasminogen activators (Cesari et al. 2010). These marketed thrombolytic agents with underlying mechanisms and dose is given at Table 17.1.

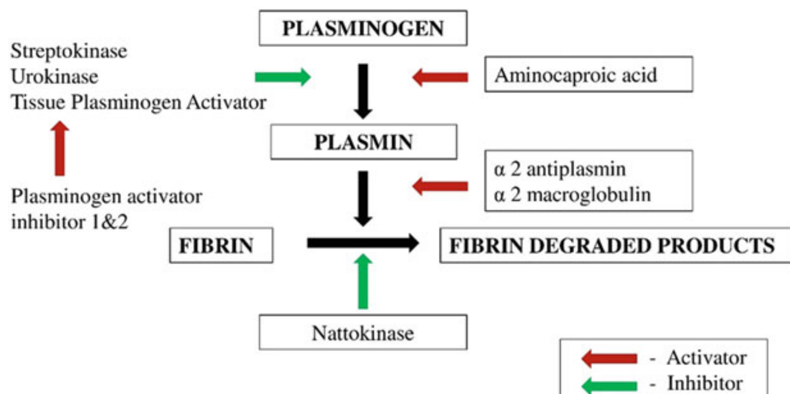

Fig. 17.1 Diagrammatic representation of fibrinolytic cascade. It depicts activators and inhibitors involved in the different steps of fibrinolytic cascade

17.2 Streptokinase

Streptokinase is a thrombolytic protease (EC.3.4.99.22) which is used to break down the blood clots in pulmonary embolism, arterial thromboembolism and myocardial infarction (Sikri and Bardia 2007). This FDA-approved drug is marketed as Streptase.

17.2.1 Source

This enzyme is mostly produced by β-hemolytic Streptococci and the bacteria containing genetic material derived from Streptococci of Lancefield group of A, C, or G (Karimi et al. 2011). The *skc* gene encoded Streptokinase is produced by different host system like bacteria, yeast. *Schizosaccharomyces pombe* and *P. pastoris* can be used for successful expression of Streptokinase (Vellanki et al. 2013).

17.2.2 Molecular Characteristics

Mature Streptokinase of 47 kDa molecular weight contains 410 amino acids, the NH_2 terminal 245 residues of this enzyme is homologous to serine proteases. From the sequence alignment of these two enzymes, it was found that the active site of streptokinase contains glycine57 instead of histidine present in serine protease (Jackson and Tang 1982).

Streptokinase mediates the conversion of plasminogen to active plasmin. It forms a highly specific 1:1 enzymatic complex with plasminogen which promotes the cleavage of Arg561/Val 562 bond in plasminogen (Young et al. 1998). The val562 binds to Asp740 of the enzymes which triggers the formation of proteolytic enzyme plasmin. Plasmin degrades the fibrin matrix of thrombus (Loy et al. 2001).

Table 17.1 Thrombolytic agents, underlying mechanisms, marketed drugs and dose

Agent	Mechanism	Marketed drugs	Recommended dose
Streptokinase	Mediates the conversion of plasminogen to plasmin	• Streptase, Sanofi Aventis Pharma	250,000 IU/30 min, IV
		• Kabikinase, Pharmacia Healthcare Ltd.	1,500,000 IU by injection
		• Shankinase, Shantha Biotechniques Pvt. Ltd.	250,000 U/vial by infusion
		• Thromboflux, Bharat Serums and Vaccines Ltd.	1,500,000 IU injection
		• Lcikinase, Abbott	1,500,000 IU injection
Urokinase	Mediates the conversion of plasminogen to plasmin	• Kinlytic, Microbix	500,000 IU IV
		• Kd-Unase, VHB Lifesciences Inc.	500,000–1,000,000 IU
		• Uropase, Cadila Pharmaceuticals	250,000 IU/vial
		• Urokinase, TTK Health care Ltd.	20,000 IU/vial
		• Dukinase, Taj Pharmaceuticals Pvt. Ltd.	25,000–100,000 IU by injection
Nattokinase	Stimulates the degradation of fibrin directly	• NSK-SD, Japan Bioscience Laboratory	100 mg (capsule)
		• Nattomax, Jarrow formulas	2000 Fibrin U/day (one capsule)
r-tPA	Breaks down plasminogen into plasmin to dissolute the blood clot	• Activase/Alteplase (Roche, Genentech)	100 mg/100 mL IV injection
		• Reteplase (Retavase), Ekr Therapeutics	10.4 U powder for IV injection
		• Tenecteplase (TNKase), Genentech Inc.	1.81 mg/mL Kit solution, IV
			50 mg/10 mL, topical

17.2.3 Enzyme Assay

The potentiality of the Streptokinase is determined by its ability to convert the plasminogen to plasmin. This is assessed by two methods.

17.2.3.1 Fibrin Clot Lysis Method (Mahboubi et al. 2012)

In this method the fibrin plate is first produced to which the enzyme is added. The activity of the enzyme is measured by the zone of lysis produced in the plate by the enzyme. To prepare fibrin plate, plasminogen is added to the agarose solution and the thrombin is added to the human fibrinogen solution (either pure form or obtained from the human plasma). These two solutions are mixed in petri dish to allow the clot formation.

The plate is allowed to be kept at 4 °C for 30 min. Then the Streptokinase (100–1000 IU/mL) is added to the plate and incubated for 8 h. The zone of lysis produced on the plate is measured which is directly proportional to the concentration of the Streptokinase. From this result, dose response curve of the enzyme can be obtained.

17.2.3.2 Chromogenic Substrate Assay (Mahboubi et al. 2012)

In this method, a chromogenic substrate is used instead of the fibrin. S-2251 (Val-leu-lys-*p*-nitroaniline · 2HCl; Chromogenix, Milan, Italy) is a chromogenic plasmin substrate. Streptokinase converts the plasminogen to plasmin which then hydrolyses the substrate. In an experimental study, the concentration of the Streptokinase used in this assay was 0.3–2.4 IU/mL. This hydrolysis is measured by the change in optical density. The rate of p-nitroaniline formation with increase in absorbance per second at 405 nm is directly proportional to the enzyme activity.

17.2.4 Production of Streptokinase

The production of Streptokinase is done by culturing Streptococcus on solid/liquid state fermentation media using different substrates at 37 °C for 24 h at pH 7. For example, *Streptococcus mutans* is grown on liquid state fermentation medium using different concentration of Corn Steep Liquor as substrate (Ghaffar et al. 2015).

17.2.4.1 Inoculum Preparation

The strain is previously cultured on agar medium. A loopful culture is transferred to the inoculum medium and incubated at 37 °C under shaking at 120 rpm for 24 h.

17.2.4.2 Fermentation Medium

It was reported that complex and nutrient rich media supported the growth of group A Hemolytic Streptococcus. Hence for successful production of Streptokinase a fermentation medium should rich in carbon, protein source, salts, etc. enriched with additional components like corn steep liquor to accomplish the nutritional requirement. The strain is incubated at 37 °C 120 rpm for 24 h at pH 7. The suitable fermentation media is composed of glucose (2 mg/L), $CaCO_3$ (5 mg/L), Yeast extract (20 g/L), KH_2PO_4 (0.5 g/L) with 0.2–1.6% corn steep liquor for optimum production of the enzyme.

17.2.4.3 Fermentation Procedure

Streptokinase used in therapeutics is generally produced from the culture of *Streptococcus equisimilis* strain H46A (Karimi et al. 2011). High yield of Streptokinase can be obtained at pH 7–7.1, pH higher than 7.8 and lower than 6.5 yields Streptokinase 25% lower than the neutral.

The strain initially grown on Trypticase soy agar, is transferred to 1 L vessel of BioF1o 110 Fermenter. At 2 h interval, the inoculum and fresh medium is added to the fermenter to maintain the fed batch culture. The pH should be maintained at 7 by adding acetic acid or NaOH. Fermentation is stopped by rapid cooling at 4 °C and by adding hexy resorcinol. The culture is then centrifuged at 10,000 rpm for 20–25 min at 0 °C for purification of the enzyme.

17.2.5 Purification

The enzyme being extracellular, should be present in the supernatant obtained from centrifugation of the culture. It is then filtered through 0.2 μm cellulose acetate filter and purified further. The protein present in the filtrate is first reduced by dithiothreitol or by β-mercaptoethanol and then the enzyme is separated from the mixture by SDS-PAGE. A high level of purification can be obtained by Plasminogen coupled to cyanogen bromide activated Sepharose 4B affinity column chromatography. Here, plasminogen is often acetylated (by p-nitrophenyl guanidinobenzoate) to increase the recovery of the enzyme. After passing the enzyme through the column, the column is washed with buffer containing Tris-Cl, NaCl and eluted with urea, Tris-Cl buffer (Babashamsi et al. 2009).

17.2.6 Production of Recombinant Streptokinase

The native Streptokinase has an antigenic capacity because of its bacterial origin. The advent of developed bio techniques allows the production of recombinant Streptokinase to reduce the antigenic capacity with increased efficacy. The Streptokinase coding region (*stk* gene) is amplified, inserted into a cloning vector (pKK223-3) and transformed into *E. coli* (strain JM105) cells (Avilan et al. 1997).

Heberkinasa, a recombinant Streptokinase is produced by expression of Streptokinase gene isolated from *Streptococcus equisimilis* in *E. coli* using high-throughput Recombinant DNA Technology. In this recombinant form, five amino acids are mutated in comparison to the native (Hernández et al. 2005).

The presence of posttranslational modification machineries makes yeast as an ideal host system for the production of recombinant protein. The recombinant vector pB2ZB2 containing the Streptokinase gene was maintained in *E. coli* DH5α and then *S. cerevisiae* (INVSc1 cells) was transformed with this recombinant vector for expression of this enzyme. The r-Streptokinase is then purified and the activity is determined (Vellanki et al. 2013).

An experimental study based on recombinant enzyme production reported the expression of r-streptokinase using Cell Free Protein Synthesis (CFPS) system based on Hela and CHO (Chinese Hamster Ovary) cell lysate. The expression vector (pT7CFE-Chis) containing the Streptokinase gene was added to the CHO cell lysate reaction mixture. Production of this recombinant enzyme was carried out in mini bioreactor equipped with expression cassette, dialysis, impeller, three probes for monitoring pH, temperature, and dissolved oxygen. Then the enzyme was purified, and the activity was quantitatively assayed by synthetic chromogenic substrate. Thus, CFPS was reported to be a promising alternative system for production of cell based therapeutic protein (Tran et al. 2018).

17.3 Urokinase

Urokinase is a serine protease (3.4.21.73) used in treatment of thrombolytic disorders like deep vein thrombosis, thrombosis of the eye, pulmonary embolism, and myocardial infarction (Masanori et al. 1985; Kunamneni et al. 2008). This enzyme is associated with extracellular matrix degradation, tissue invasion, tumor cell migration and metastasis (Danø et al. 1985).

17.3.1 Source

Major sources of Urokinase are human urine, blood, and extracellular matrix of many tissues. Further studies reported vascular endothelial cells, smooth muscle cells, fibroblasts, epithelial cells, monocytes, and tumor cells of different origin as other sources of this enzyme (Sobel et al. 1952; Clowes et al. 1990; Eaton et al. 1984).

17.3.2 Molecular Characteristics

Urokinase synthesized as single polypeptide consists of 411 amino acids. This enzyme of molecular weight 53 kDa contains three domains—N terminal growth domain which bind to the Urokinase receptor, C-terminal catalytically active protease domain where the active site contains His204, Asp255, and Ser356, and Kringle domain involved in interaction with plasminogen activator inhibitor (Holmes et al. 1985; Bansal and Roychoudhury 2006). It has a unique glycosylation site at Asn 302. The zymogen or inactive form of this enzyme is converted into its active form by cleaving at Lys158-Ile159 bond resulting in, formation of two chains connected by disulfide bond (Fig. 17.2).

Disulfide bridges in the catalytically active domain are essential for sustaining the fibrinolytic and amidolytic activity. Like Streptokinase it cleaves the Arg-Val bond.

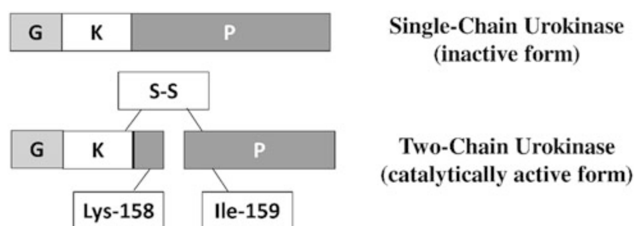

Fig. 17.2 Structure of single-chain and two-chain urokinase forms. *G* growth factor-like domain, *K* kringle domain, *P* protease domain. Single chain urokinase is cleaved into two chains to form active urokinase (Stepanova and Tkachuk 2002)

17.3.3 Enzyme Assay

Measurement of Urokinase activity is essential for studying the thrombolytic therapy of urokinase. The activity of the enzyme can be determined either by direct hydrolysis of chromogenic substrate S-2444 or by converting the plasminogen to plasmin.

17.3.3.1 Enzyme Assay by Using S-2444

S-2444 is a synthetic substrate which has a tripeptide sequence pyro Glu-Gly-Arg-pNA specific for the enzyme. For this assay the enzyme is first incubated with Tris-Cl buffer and Triton-X at 37 °C for 10 min. Then the substrate S-2444 is added to it. The enzyme activity can be determined by measuring the change in absorbance at 405 nm (Svoboda et al. 2004).

17.3.3.2 Assay Using Plasminogen and S-2251

In this assay, the enzyme is incubated with Tris-Cl buffer and Triton-X at 37 °C for 10 min followed by the addition of plasminogen and the substrate S-2251 respectively. S-2251 is a plasmin substrate. After activation of plasminogen to plasmin by the enzyme, it hydrolyses the chromogenic substrate S-2251 which can be measured by monitoring the change in absorbance at 405 nm. Thus a linear relationship can be established between the concentration of Urokinase and the change in absorbance (Svoboda et al. 2004).

17.3.4 In Vitro Production of Urokinase

Urokinase isolated from the tissue culture and urine is costly which limit its use as therapeutics. To improve the production of Urokinase different cell lines viz. yeast, bacteria, insect, plant, and mammalian cell lines are used. Various cell lines employed for the production of Urokinase includes Chinese hamster ovary cell of CHO, *Saccharomyces cerevisiae*, HT 1080 kidney cell, *E. coli* cells, Mouse cells LB6 yielding the enzyme activity of 860 pU/mL, 1863 pU/mL, 140 pU/mL, 1500 pU/mL, 0.8 mg/L/day, respectively (Kunamneni et al. 2008).

Due to the lack of posttranslational modification system in prokaryotes, the mammalian cell lines are mostly favored for the production of the Urokinase. But in case of mammalian cells the production of enzyme is less due to slow growth rate, hence the cells should be cultured for prolonged time. Most of the mammalian cells lines are anchorage dependent i.e., solid surface is required for them in in vitro growth. Micro carrier beads are used for the culture of these cells.

17.3.4.1 Media Used for Production

Production of Urokinase from the mammalian cells cultured in DMEM with 10% fetal bovine serum is used. The production of this enzyme is enhanced with the reduction of serum content. For Urokinase induction, preferable compounds are saccharides viz. glucose, inositol, ribose and deoxyribose, hormones such as

adrenaline (Bansal et al. 2007). Arginine is also used as inducer as it is the precursor of nitric oxide (Ziche et al. 1997).

Roychoudhury and colleagues, used HT1080 kidney cell line for production of Urokinase in T flasks and bioreactor. To achieve the substantial growth, cells are immobilized into micro carrier beads (100–300 μm). Polysaccharide gel, Porous polyurethane matrices, glass fiber packed columns are used for immobilization. For reduction of serum from 10% to 2% the media is supplemented with 10% (w/v) glucose, 1% (w/v) beef extract, 1% (v/v) non-essential amino acid mixture, and 0.1% (v/v) vitamin B12 solution. Alkaloids or agents like pronase are used for the stabilization of release of the enzyme (Roychoudhury et al. 1999).

17.3.4.2 Production and Purification of Urokinase

The HT1080 kidney cells used for the production of Urokinase are initially grown on DMEM containing 10% FBS. Then the micro carriers are added to it to immobilize and allowed to grow those microcarrier-coated cells on medium containing less amount of FBS (<10%). The agitation speed should be maintained at 50 rpm to minimize the hydrodynamic effect. To achieve maximum Urokinase activity, bioreactor operation should be carried out for 65 h. Then the cell culture extract is filtered and further purified. One step purification is always preferred to reduce the loss of product occurred in case of multistep purification. For different cell lines different purification system is applied (Table 17.2). For example: Sepharose column chromatography, Cu II IDA polyacrylamide cryogels chromatography are used for the purification of this enzyme from HT1080 kidney cells (Bansal et al. 2007; Khaparde and Roychoudhury 2005; Kumar et al. 2006).

Table 17.2 Different purification techniques of urokinase from different cell lines (Bansal and Roychoudhury 2006)

Culture broth	Technique	No. of Steps	Fold purification	Enzyme activity (% recovery)
HT1080 cells	Cu(II)-IDA polyacrylamide cryogel chromatography	1	27	80
Recombinant CHO cells	Anti-sc-u-PA sepharose 4B chromatography	1	50	90
Mammalian cells	Carboxylated polyethyleniminopropyl trimethoxysilane chromatography	1	–	91
Mouse sarcoma 3T3 cells	Affinity chromatography, anion exchange chromatography, gel filtration	3	–	32

17.3.5 Production of Recombinant Urokinase

Recombinant studies of Urokinase was performed in different host system.

The recombinant human urokinase-type plasminogen activator or u-PA with 6× his tag at C terminus in was produced in *E. coli* cells. The activity of this recombinant enzyme remains same as that of the native after refolding (Tang et al. 1997). On the other hand, yeast (*Saccharomyces cerevisiae* MC16 strain) was used as efficient expression system for recombinant Urokinase production. Yeast 2μ derived plasmid pJB207 was used as a cloning vector which contains the pro-Urokinase gene, Gal7 promoter, Gal10 terminator, and MPR gene. Large amount of human pro-Urokinase were produced and its mutants as core glycosylated form accumulated in endoplasmic reticulum of cells were converted into its biologically active form by denaturation-refolding process (Hiramatsu et al. 1991).

CHO was also reported as an ideal host for the production of recombinant enzyme as it can be easily genetically manipulated and it has a modification system for producing the glycosylated protein (Warner 1999). A recombinant CHO cell line MGpUK was used as host where pro-Urokinase gene was cloned into pcPUK expression plasmid and co-expressed with pDCH1P plasmid for screening and gene amplification (Kim et al. 2001).

17.4 Nattokinase

Nattokinase is a serine protease (EC.3.4.21.62) used to reduce the blood pressure in arteries and valves and to treat stroke (Dabbagh et al. 2014) by dissolving blood clots. This enzyme is found from the fermented soybean food called "natto" in Japan. It is extracted from the solid-state fermentation of soybean curd residue (SCR) and wet distiller grain (WDG) (Zu et al. 2010).

Nattokinase is mostly favored in treatment of embolic disease and CVD such as hypertension, angina pectoris, high blood cholesterol, deep vein thrombosis, atherosclerosis, hemorrhoids, varicose vein and peripheral artery disease (Chen et al. 2018). Because of its safety, low cost, and natural supplements. This enzyme is safe for oral administration which increases fibrinolytic activity in the plasma after intraduodenal absorption. Low substrate specificity and hemorrhagic side effects of Urokinase and Streptokinase and short half-life of tissue plasminogen activator limit their use in treatment of CVD.

17.4.1 Molecular Characteristics

Nattokinase consists of 275 amino acids except cysteine and have molecular weight of 27 kDa. It has a sequence similarity with subtilisin E. Primary structure of this enzyme contains a conserved region comprising 29 residues of signal peptide and 77 residues of propeptide (Nakamura et al. 1992).

This enzyme degrades the fibrin and plasmin substrate H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride (H-D-Val-Leu-Lys-pNA, S-2251). It does not activate the plasminogen directly rather it cleaves the plasminogen inhibitor and activate other plasminogen activator like pro-urokinase, tissue plasminogen activator (Fujita et al. 1993). The fibrinolytic activity is fourfold higher than the plasmin (Sumi et al. 1987).

17.4.2 Enzyme Assay

The fibrinolytic efficiency of the Nattokinase can be determined by its ability to lyse the fibrin clots (Zu et al. 2010). The enzyme activity can be assessed by two ways.

17.4.2.1 Fibrin Plate Assay

Fibrin plate is prepared by mixing the fibrinogen solution with thrombin and is spread over the plate. The plate is heated at 85 °C for 30 min. Then the Nattokinase is added to the solution. The zone of fibrinolysis is proportional to the concentration of the enzyme.

17.4.2.2 Assay of Nattokinase by Measuring the Change in O.D.

In this assay, Nattokinase sample is added to the mixture of fibrinogen solution and thrombin and incubated for 20–40 min. Then the reaction is topped by adding trichloroacetic acid. The fibrin obtained from the lysis of fibrinogen by thrombin is again cleaved by the Nattokinase. Thus, the change in absorbance caused by the fibrin lysis is directly proportionate to the concentration of the Nattokinase.

17.4.3 Production of Nattokinase

17.4.3.1 Fermentation Conditions

Bacillus subtilis natto B12 strain is isolated from the natto food for the production of Nattokinase. Initially the strain should be cultured in the starter medium containing 1% peptone, 0.5% beef extract and 0.5% NaCl for 24 h at 37 °C. The pH of the media should be around 7. After the cultivation, 2% (v/v) culture should be transferred to 50 mL fermentation media having 2% maltose, 3% soybean meal, 0.5% NaCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.4% K_2HPO_4, and 0.1% KH_2PO_4 (Wang et al. 2009).

Nattokinase is active at both neutral and alkaline pH. The optimum activity of the Nattokinase is at pH 8. At extremely high and low pH, the activity is decreased. Within the pH range 6–9, more than 80% activity is retained which rapidly lowered to 20% at pH 12 with loss of activity at pH 4. This enzyme is stable at the temperature range of 30–50 °C exhibiting optimum activity at 40 °C. Enzyme activity is shown to be lowered with the increase in temperature. The activity of this enzyme is increased after 40 h of incubation and is maximum at 60 h of incubation with agitation at 180 rpm.

17.4.3.2 Extraction and Purification of Nattokinase

The sequential steps for extraction and production of Nattokinase are as follows (Wang et al. 2009)

1. After the incubation, the culture media should be centrifuged at 8000 rpm for 20 min at 4 °C.
2. Then the supernatant containing crude enzyme is purified by following steps. All the purification steps are performed at 4 °C.
3. Addition of ammonium sulfate to the supernatant for salting out of proteins.
4. Collection of the precipitate by centrifugation followed by the dissolution of pellet in Barbitone Sodium Chloride buffer (BSC).
5. Dialysis of the solution against the same buffer for 24 h.
6. Purification of the enzyme dialysate through the Sephadex G-75 column chromatography.
7. Elution of column with BSC at a flow rate 0.5 mL/min and the collection of fractions of elute with high enzymatic activity for further purification.
8. Fraction with high enzyme activity is again passed through the phenyl-Sepharose 6 fast flow column chromatography.
9. Elution of column with gradient of ammonium sulfate solution.
10. Collection of the fraction containing high concentration of enzyme followed by ultrafiltration and removal of the ammonium sulfate to obtain purified enzyme with specific activity of 5316.36 IU/mg, 43.2% yield, and 56.1-fold purification.

17.4.4 Production of Recombinant Nattokinase

To enhance the production and stability of recombinant Nattokinase, *E. coli*/*Bacillus subtilis* shuttle vector is used. The shuttle vector is obtained from the fusion of plasmid pUC18 (*E. coli* DH5α strain) and plasmid pUB110 (*Bacillus subtilis* WB700, original strain—*Staphylococcus aureus*). But due to production of high structural instability, the pUC18 is replaced with R6K. The fusion of pUB110 and R6K followed by the amplification and restriction digestion results in production of hybrid plasmid pUBCII, pUKX. The NAT gene (aprN) is inserted into that hybrid plasmid to form pUKVI-NAT2 plasmid. *Bacillus subtilis* bearing this plasmid is then employed for large-scale production of recombinant Nattokinase in an effective way (Chen et al. 2007).

In an experimental study, it was reported that production of recombinant Nattokinase from *Bacillus subtilis* was increased in the presence of casamino acids, asparagine or glutamine but decreased in presence of glucose. Even the production was increased up to fourfold in media supplemented with glutamate and metal ions (Chen and Chao 2006).

A current research also reported the production of Nattokinase from *Pseudomonas aeruginosa* CMSS where the activity of the enzyme to lyse the blood clots is much higher in the UV radiated strain than the wild type. Further work on efficient

production of Nattokinase in an inexpensive way has been reported by Chandrasekaran and group (2015).

17.5 Tissue Plasminogen Activator (tPA)

Human tissue plasminogen activator (tPA), an enzyme belongs to serine protease family, is an essential component of fibrinolytic system of blood vessel endothelial cells. The primary function of this enzyme is to dissolve the blood clots formed by fibrin meshes by the activated plasminogen. tPA mainly catalyze the conversion of activated plasminogen to plasmin, thus facilitates the thrombolysis. The role of tPA in extracellular matrix regulation is also reported wherein it is stated that tPA induces matrix degradation, the excessive matrix degradation may promote tissue fibrosis (Lin and Hu 2014).

The advancement of biotechnology, makes it possible to develop recombinant tPA for therapeutic purpose. The clinical indications for use of this enzyme include ischemic heart disease, myocardial infarction, pulmonary embolism, etc. Drugs manufactured using the synthetic tPA are: Alteplase, Reteplase, and Tencteplase. Among them, Alteplase is the composed of normal human plasminogen activator without any modification whereas rest two are modified form. To reduce the drug-induced side effects including nausea, bleeding, vomiting, dizziness, these drugs have undergone modifications which further amplify their pharmacokinetic modification with high specificity (Collen and Lijnen 2009).

17.5.1 Mechanism of Action

The human tPA, a glycoprotein of 69 kDa molecular weight, comprises of 527 amino acid residues including 35 cysteine residues with 17 disulfide bonds. It contains five functional domain—N terminal finger domain or N domain, Epidermal growth factor-like domain or E domain, two Kringle domains or K1 and K2 domain, Protease catalytic domain or P domain. P domain or the catalytic domain contains the His322, Asp371, and Ser478 within the active site essential for enzymatic activity. The F domain and K2 domain mediates the binding of this enzyme to fibrin required prior to the plasminogen activation (Collen and Lijnen 2009).

The thrombolytic mechanism of tPA lies on the activation of plasminogen to plasmin to breakdown the crosslinking of the fibrin mesh made in the blood clot. The sequential activity of this enzyme includes binding to the fibrin on clot surface followed by activation of plasminogen. For the activation, this enzyme binds to plasminogen and cleaves at Arg561-Val562 position in the peptide to yield plasmin. This plasmin subsequently acts on fibrin network to dissolute the blood clot. The Plaminogen Activator Inhibitor 1 or PAI-1 is shown to inhibit the tPA activity by binding to the Lys296-Arg299 region of its peptide (Jilani and Siddiqui 2019).

17.5.2 Enzyme Assay

The thrombolytic activity of the enzyme is evaluated by the assessing its ability to cleave plasminogen on agarose-fibrin plate. The agarose-fibrin plate could be prepared by adding plasminogen, thrombin, and fibrinogen to 1% agarose gel dissolved in normal saline water at 45–55 °C. Then the plate was then incubated with sample for 24 h at 37 °C. The activity of the enzyme can be measure from the clear zone obtained on the fibrin plate (Long et al. 2015).

17.5.3 Production and Purification of tPA

The production of tPA in industry scale for the therapeutic uses requires the functional preparation of this enzyme. Different prokaryotic and eukaryotic systems such as Chinese hamster ovary cells, human uterus, *E. coli*, even recombinant host such as yeast and insect system are used for the expression and purification of this enzyme. Due to low cost and rapid growth, *E. coli* are the most preferable system for expression of most eukaryotic proteins in large scale.

To achieve high-throughput protein expression, *E. coli* system undergoes various modifications.

Xiaobin long's team developed a method to yield high level of purified recombinant tissue plasminogen activator protein in *E. coli* system by autoinduction (Long et al. 2015).

17.5.3.1 Bacterial Strain and Growth Conditions

In a study conducted by Datar, *E. coli* strains K12 (DE3) were used for recombinant protein expression (Datar et al. 1993). The competent cells were prepared chemically and then transformed using standard protocols (Novagen, USA). Here, Luria Bertani broth media is used for the normal growth of the strain. For primary culture, bacteria were allowed to grow on 5 mL LB media and incubated at 37 °C.

17.5.3.2 Construction of Expression Vector

In this study, the *tpa* gene obtained from the human liver was amplified by PCR with the primer containing restriction sites. Then the amplified gene was sub cloned to pXL130 expression vector.

17.5.3.3 Expression of Recombinant tPA Protein

The chemically competent *E. coli* cells were transformed with the fused expression vector pET28a and were allowed to grow on media containing tryptone, yeast extract, NaCl, glucose similar to the composition of LB broth.

17.5.3.4 Fermentation Mode and Size

This production is carried out in batch culture for 1–2 days. The size of the fermenter is 1700 L.

17.5.3.5 Purification of tPA Protein

The strategy of protein purification is almost same for most of the enzymes. The sequential steps for the extraction and purification of tPA is as follows-

1. Harvesting of cells by centrifugation at 4000 rpm for 10 min.
2. Resuspension of bacterial pellet in ice cold lysis buffer (Tris-CL, pH 8) followed by sonication (3 s on–off cycle, 30% amplitude) for 20 min to lyse cells.
3. Centrifugation at 15,000 *g* for 30 min at 4 °C to remove cellular debris.
4. Solubilization of bacterial inclusion bodies followed by ultra filtration.
5. Sulphonation of the solution by Na_2SO_3, followed by ultrafiltration again.
6. Separation of tPA from solution by ion-exchange chromatography and then the elute is further subjected to Lysine chromatography.
7. The elute obtained from the above step further subjected to the size exclusion chromatography.
8. Collection of final eluate containing pure tPA, filtered using sterile filter, combined, and concentrated.
9. The purified protein obtained would be quantified and subjected to SDS-PAGE and Western Blotting for determining purity of the protein.
10. The final concentration of the product was 460 mg/L and the overall yield was 2.8%.

Although *E. coli* is most widely used expression system, the unavailability of the systems required for posttranslational modification and proper folding of eukaryotic proteins in bacterial system make its use limited. To overcome this problem, yeast and CHO cell lines are mostly used as expression system using different culture conditions and purification methods (Table 17.3).

Table 17.3 Culture conditions and techniques employed for production of recombinant tPA

Expression system	Expression vector	Culture conditions	Purification method	Reference
Aspergillus niger	pBLUE-AmdS-PyrG, CYPB	• Culture media: CAS-AM medium containing glucose, KCl, casamino acid, $NaNO_3$, KH_2PO_4, $MgSO_4$, $CuSO_4$, $FeSO_4$ • Growth Temperature: 20–30 °C • Incubation period: 48 h • Rotation: 125–200 rpm • pH: 5.5–6 • Type of culture: Fed Batch • Agitation: 1000 rpm	Ni-NTA column chromatography, ion-exchange chromatography	Wiebe et al. (2001)
CHO cell line	pXL261	• Fermentation mode: Batch • Fermentation duration: 5–7 days • Product Concentration: 33.5 mg/L • Product yield: 47% • Fermentor size: 7000 L	Microfiltration, ultrafiltration, gel chromatography, affinity chromatography	Datar et al. (1993)

17.6 Future Directions and Conclusions

Since the dawn of medical sciences, researchers have been on an ever-expanding quest to find more efficient tools to combat diseases and improve the quality of living. The, discovery of thrombolytic enzymes and their role in treatment of cardiovascular diseases (CVD), emerged as a significant advancement in the field of medical sciences. As per the recent statistics, by the World Health Organization, 17.9 million fatalities are due to CVD, annually. This massive human fatality every year demands an urgent, large-scale and efficient production of thrombolytic enzymes, that a promising cure, saving thousands of lives. However, since, the traditional production methods are unable to meet the demands, more recombinant strains are being engineered to speed up the process and make large-scale production relatively hassle-free.

Thrombolytic enzymes, like Streptokinase, Urokinase, Nattokinase, and tPA have been demonstrated to be a potential candidate in therapeutic treatment of fatal diseases like coronary embolism, myocardial infarction, ischemic stroke, etc. The future of discovery and production of novel thrombolytics, lies in the identification of novel enzymes of microbial origins, and using, carefully engineered microbial consortium, to increase the net production efficiency. Several microbes isolated from food or non-food sources, have been reported to be a promising source of thrombolytic enzymes, *Bacillus* sp. being the most popular one (Kotb 2014). Interestingly, fermented Asian food has been reported to provide a suitable habitat for these microbes to produce thrombolytic or fibrinolytic enzymes (Kotb 2013). Moreover, some studies have also demonstrated the presence of thrombolytic enzymes in vampire bats, snakes, earthworms, and in plant lattices (Mihara et al. 1991; Krätzschmar et al. 1991; Gao et al. 1998; Islam et al. 2016).

A primary problem encountered in the industrial production of any biomolecules is the gradual loss in productivity of the strain over extended time. A possible reason behind this could be the fact that newly introduced genetic manipulations to the production strains are often not favored by natural selection. Hence, there is a gradual loss in the desired phenotype. In a recent publication, Giri and colleagues addresses this issue and suggests that this reduction in productivity could be associated with the eco-evolutionary history of the strain that is often ignored (Giri et al. 2020). It has been already established in several studies, that cell-to-cell communication and community diversity has a significant impact on the proteome and metabolome of an organism (Davenport et al. 2015; Noecker et al. 2019; Sedlacek et al. 2016). Thus, exploring the eco-evolutionary history of a novel or a genetically manipulated strain that can be potentially used for industrial production, could be the missing link that can lead us into a new era of industrial production of fibrinolytic enzymes.

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Enzymes in Textile Industries

18

Vartika Verma, Sunanda Joshi, Monika Choudhary, and Nidhi Srivastava

Abstract

The existence of enzyme was reported since half of nineteenth century and was broadly used in various industrial processes. Due to its specific nature, it finds numerous applications in various fields. In the field of industrial enzymology, enzyme role in various processes in textile industry is swiftly growing on a huge scale. In textiles, they have been much preferred due to its biodegradable nature, non-toxicity and environment-friendliness. Degradation of lignin, degradation of hydrogen peroxides and bleaching textiles are the main focus areas in the textile industry that are enzyme-induced process. Popular and most frequently used biocatalyst in the textile industry is amylases, peroxidase, and catalase. Enzymes (biocatalysts) are budding in a huge way in the field of textile. There still is significant prospective for innovative and enhanced enzymes applications in potential textiles. The current review focuses the role of various enzymes and their applications in the fields of textiles.

Keywords

Enzymes · Textile industry · Eco-friendly

18.1 Introduction

Enzymes have achieved a huge recognition in various industrial sectors, mainly in the textile industry due to their following nature: non-toxic, environment friendly, and stereo specific. They are virtually valid to all industrialized ladders in textile process. Various enzymes like cellulases, proteases, and Amylases are significant to

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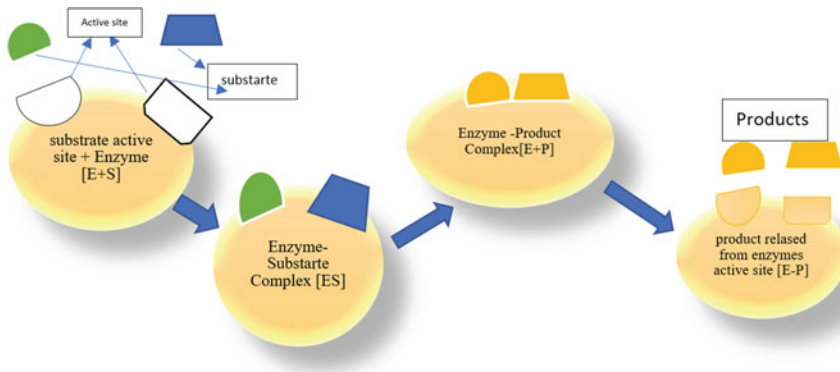

Fig. 18.1 Action of enzyme and substrate

mention, for the various steps of textile process. However, disregarding the huge potentials theses enzyme face various limitations to be worked on industrial sector due to conditions like pH and temperature that are engaged in textile processing that reduce properties and action of their work. With the growth and progression in the enzymology various new enzymes are being identified and modifications in existing enzymes are also carried out to make use of enzyme in textiles (Madhu and Chakraborty 2018).

Enzymes were initially originated from "Enzymos" which is a Greek word means "from the cell." They are a chain of more than 250 amino acids and are superior protein catalysts that catalyze unambiguous chemical or biochemical reactions. Figure 18.1 below shows the action of the enzyme. They have vastly precise active sites present in the molecule that implement catalytic reaction. A variety of simpler compounds could be replaced with the enzymes that can imitate the identical behavior similar to that of biocatalysts and can escort for improvement of reaction rate, which can help to reduce costs and augment the enzymatic process.

They are grouped or clustered based on specificity. Enzymes have various salient features as they have been a highly specific nature of the reaction, easy going condition, safe, and non-corrosive. In this process, requirement of energy and chemicals, is less hence they are easily biodegradable. Enzymes under unfavorable condition remain unaffected, but the physical configuration may get tainted.

In Japan during 1989, the perception of using enzyme with fabric in order to augment their surface properties was budded. This method was well thought-out as imperative as it was hygienic and eco-friendly to the environment. When utilization of enormously specific catalysts is a prerequisite, enzymes are finding great applications in the chemical and other industries. However, the application of enzymes in general is restricted to fewer numbers of reactions as they lack stability in various organic solvents and at high temperatures. As an outcome, various attempts are made by scientists to craft new enzymes with novel properties (cdesign@cdesign.web.id 2015). Due to constant escalating noxious waste level in countries, governments are discouraging restrictions on release of toxins/poisons.

Therefore, increasing requirement for hygienic processes is further stipulated. Textile industry involves exploitation of diverse hazardous chemical processing sector, which shares foremost contribution to widespread pollution. So the use of assorted enzymes unaided or in amalgamation of additional enzymes plays key role as a substitute processes (Mojsov 2011). The relevance of biocatalyst in textile processes has gathered much curiosity due to a variety of compensation of enzymes like harmless, ecological and environmentally friendly, so it does not pretend any harm to the people or the vegetation around. It also helps in speeding up the process (Chooromoney 2018). There are various processes in the textile which can securely make use of enzymes like desizing, scouring, bleaching, dyeing, and finishing; on the other hand, the steady use of the traditional chemicals is creating lots of fumes of emissions when laid-off into the environment. In the field of enzyme technology, the capabilities of solitary enzymes or enzyme mixtures for specific applications have been explored to a great extent to make it recipient for the textile industry. As today there are lots of precincts on the industries in order to sustain the environment, usage of enzymes appears to be a tremendous substitute to trim down pollution to some coverage. Some of the biocatalysts used in the textile industry are laccase, amylases, catalase, etc. (Uzzal 2013).

The various known enzymes that are employed for fabric preparation and finishing include:

- Hydrolases
- Oxidoreductases

Enzymes have benefited the textile industry as they do not produce any toxic effluent, can be effortlessly deactivated and disposed off easily and are measured to have promising future in textile industry. For various chemical or biological reactions, enzymes act as catalysts. In comparison to the common chemical catalysts being used, enzymes proved to be more proficient and the reaction rate boosted by many folds. They have the additional advantage to undergo a reaction under gentle conditions like frigidness, neutral aqueous solution, and optimum pressure (Uzzal 2013).

18.1.1 Salient Features of Enzyme Useful in Textile Production

- After completion of the reaction, the residue containing enzyme is disposed of easily without causing harm to the environment, i.e., non-toxic and therefore it is eco-friendly and biodegradable.
- Proved to be an appropriate substitute for polluting chemicals.
- Various toxic and hazardous chemicals in textile processes which used that resulted in a lot of pollution but now the appropriate substitute of such chemicals is enzymes that have made the recycling/treatment cost of the waste water to be minimized.
- Speed up/accelerate the process.

- The substitutes in the textile need very high specificity and enzymes are extremely specific and catalyze only specific substrates.
- Enzymes work in placid conditions.
- Easy controllability.
- Very susceptible.
- Enzymes can compute very tiny concentrations of substances.
- Enzymes can be easily reused.
- Limited usage of water and usage is bridged by almost 19,000 L per ton of textile bleaches.
- Since enzymes are expensive its usage becomes limited.
- Usage of enzymes, makes it time consuming and expensive.

18.2 Various Enzymes Used in the Textile

In today's world, the use of enzymes in the textile chemical process has been recognized worldwide. There are a variety of enzymes used in the textile industry to avoid pollution to the environment. Some of them are amylase, cellulose, catalase, laccase, peroxidase, etc. Some of the enzymes used in textile industry is as depicted in Fig. 18.2.

18.2.1 Cellulase

In today's scenario, cellulases are one of the unbeaten enzymes used in textile industry. Conventional stonewashing made use of amylase and dealing of jeans with the pumice stone (Kuhad et al. 2011). It is a multitalented enzyme that can be used efficiently during textile processing to substitute for chemical treatments. In terms of environmentally and economically friendly also for energy consumption, they are more acceptable and more suitable for use within the textile industry (Simic et al. 2015). Cellulases have been lucratively used for the bio-stoning of jeans and biopolishing of cellulosic fabrics. During this practice, cellulases work on the cotton

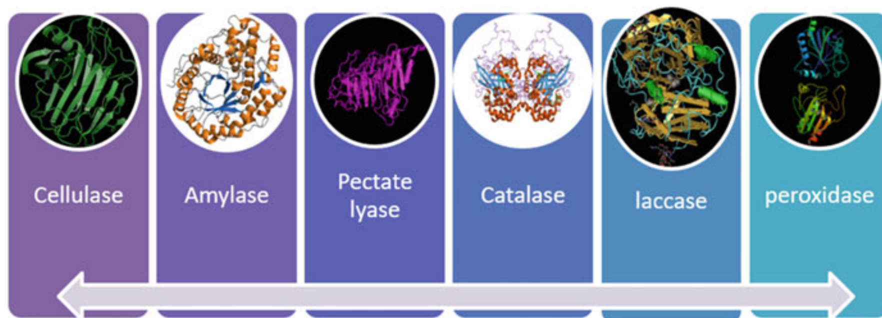

Fig. 18.2 Various enzymes used in textiles

fabric and shatter off small fiber ends on the yarn surface, thereby loosening the dye, which is easily detached by mechanical abrasion in the wash cycle (Kuhad et al. 2011). A special application of cellulases is used for the world-famous “stone wash” and also for many other lesser-known industrial applications but not less important (Simic et al. 2015).

18.2.2 α-Amylase

Desizing is an imperative step in the textile industry and amylases enzymes remain the foremost preference in textile industry to smooth the evolution of the confiscation of the starch-containing size that has served as a shielding coating on yarns. From moment in time to time scouring and bleaching steps are united with the desizing processing to trim down processing costs (Araujo et al. 2010). Sizing agents like starch are applied before fabric production to make sure a hasty and safe weaving practice and it becomes the most important choice in textile industry for desizing process (Souza 2010).

18.2.3 Pectate Lyase

Bioscouring is a newfangled procedure for elimination of various molecules like pectin, proteins, and waxes from raw cotton with an association of a variety of enzymes. A range of enzymes like lipases, cellulases, and proteases has been tried for the route, but the use of pectinases in bioscouring process offers recompense over use of other enzymes. Most recent bioscouring processes utilize alkaline pectinases with pectate lyase activity (Calafell et al. 2005).

18.2.4 Catalase

Today, most common step in textile industry is bleaching of textile fabric or yarn. The bleaching is facilitated by hydrogen peroxide. Catalase is one of the frequently used enzyme that aids in breaking down of hydrogen peroxide and results in formulation of nascent oxygen and water. It is utilized for bleach process in textile industry. Once the bleaching step is completely done, remaining hydrogen peroxide is left out in the container and complete removal of Hydrohen peroxide is necessary for dyeing process. Unfinished or partial removal of hydrogen peroxide is inefficacious and outcomes in poor dyeing which result in discrete alter in color, shade, strength as well as inconsistent dye circulation. Therefore, eradication of the enduring hydrogen peroxide is obligatory step (Enzymes 2015).

18.2.5 Laccase

It is one of the promising enzymes that can reinstate the conformist chemical processes in the textile industry. However, due to derisory enzyme stocks the use of laccase on marketable degree is limited. Thus, efforts are made in order to accomplish economical overproduction of laccase. The amendments are made by various pathways to achieve more vigorous and dynamic biocatalyst. Another supplementary problem is the toxicity of redox mediators and cost. Further inquiry should consider about different and less contaminating mediators such as the natural mediators produced by laccase in a bio-environment during lignin degradation (Couto and Toca-Herrera 2006).

18.2.6 Peroxidase

The enzyme peroxidase is the well-known biocatalyst used in the textile industry that helps to decolorize textile effluents, also it has great competence to eradicate aromatic amines and phenolic compounds from aqueous solutions. A prospective enzyme which is horseradish peroxidase (HRP) has contributed a lot in the blanching of textile dyes and effluents. The enzyme requires optimum conditions for its effective performance. There are a few important factors to be maintained for effective working of the factors such as pH and the amount of H_2O_2 (Forgiarini and de Souza 2007).

18.3 Enzyme Utilization During Textile Process

There are various steps in the textile processing that involves usage of enzymes. Figure 18.3 gives a brief description of the steps that utilize enzymes during textile process. Figure 18.4 shows various treatments given to natural fibers.

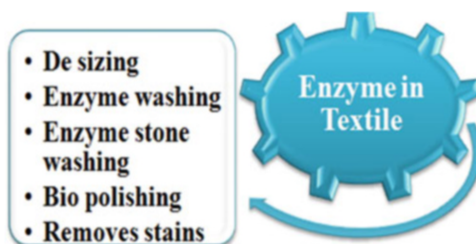

Fig. 18.3 Steps in textile processing

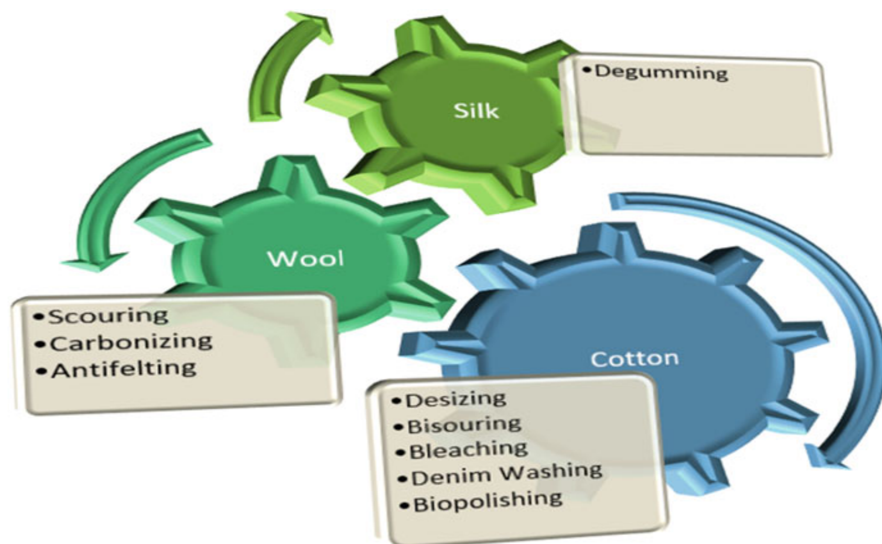

Fig. 18.4 Enzymatic treatment given to natural fibers

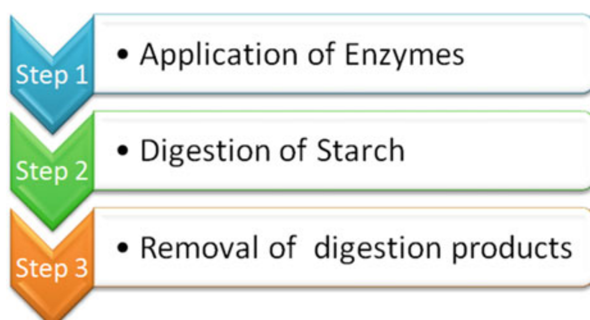

Fig. 18.5 Steps involved in desizing

18.3.1 Enzymatic Desizing

α-Amylase is a hydrolytic enzyme that splits starch into maltose and dextrin. The benefit of these enzymes is their precision for starch and removing it without destruction. α-Amylase is preferred for desizing processes at temperature (30–60 °C) and pH is 5.5–6.5 and hence amylases are used to remove starch for better and standardized wet processing in the textile industry. There are three main steps involved in this process. Figure 18.5 shows steps involved in desizing.

18.3.2 Enzymatic Scouring (Bioscouring)

The scouring process involves amputation of synthetic material present on the cotton exterior amalgamation of enzymes viz., cellulase and pectinase are used for bioscouring. Figure 18.6 shows a process involved in conventional scouring. During this process, pectinase helps to devastate the cotton cuticle by flouting pectin and removing the interconnection between the cuticle and cotton fiber, whereas cellulase assimilates the primary wall cellulose. Management is soft during the enzymatic scouring and in alkali scouring it is harsh (Madhu and Chakraborty 2018). Enzymatic scouring lowers the exposure to health risks as there is no use of aggressive chemicals. A typical enzymatic bioscouring is shown in Fig. 18.7.

18.3.3 Enzymatic Bleaching

The blanching of natural pigments to impart a pure white/pale appearance of the fibers is termed as bleaching. Most frequent industrial bleaching agent is hydrogen peroxide (H_2O_2). Soon the substitute of hydrogen peroxide by an enzymatic bleaching system has led to superior product quality. This also helped inconsiderable investments on water needed for the confiscation of hydrogen peroxide. Using an

Fig. 18.6 Conventional souring process

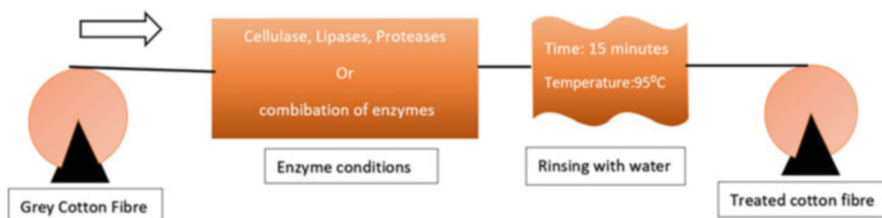

Fig. 18.7 Typical enzymatic souring process

amalgamation of suitable enzyme systems like pectinases and glucose oxidases that activate on optimum active pH and temperature range proves to be superlative option. Bleaching effect on cotton fabrics using laccases in low concentrations was cited in various literatures. The short period exposure or enzymatic pre-treatment proved to be sufficient for improved fabric whiteness. With advancement in the field of enzyme technology various new methods proved to be effective in enzymatic bleaching. The enzymatic process results in compact water expenditure, reduced energy and time utilization compared to the traditional methods (Uzzal 2013).

18.3.4 Biopolishing

Biopolishing technique for the ultimate treatment of cellulosic fabrics with cellulase enzymes was first cited by Novo Nordisk. The main focus was to improve the eminence of the fabric by eliminating the extra fibers from the surface and making it flexible and even.

In the traditional process, final touch is done by chemical treatment, also the methods used are temporary and fuzz is formed. Figure 18.8 illustrates immobilization of enzyme prior biopolishing. Once fibers chemicals are detached, it led to destruction of surface and appearance of the fabric, whereas biopolishing is a type of permanent treatment which keeps the fabric in good condition after several repeated washing and also quality of the product is enhanced.

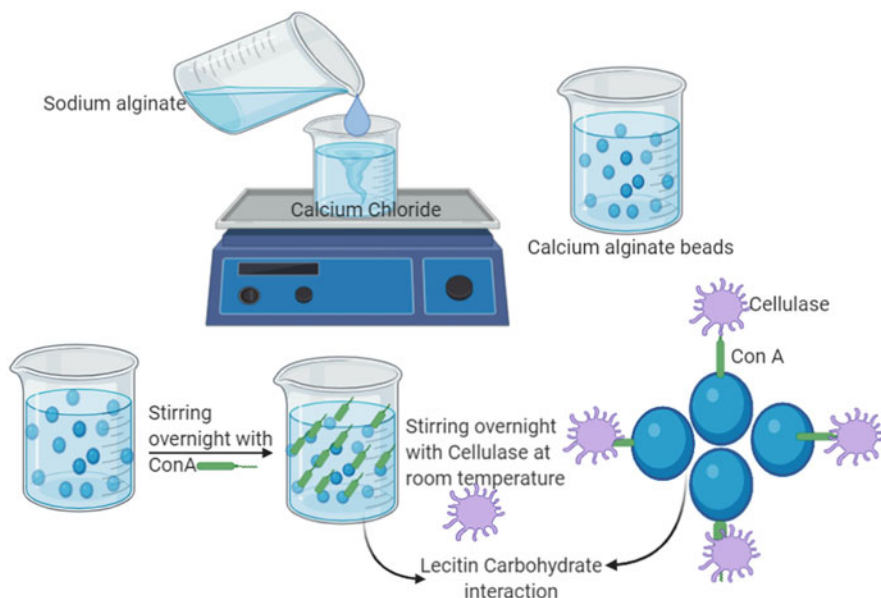

Fig. 18.8 Immobilization of enzyme prior biopolishing

18.3.5 Enzymatic Treatment to Denim

A well-known heavy grade cotton is denim onto which the dye is mainly adsorbed over the surface of the fiber. In conventional process, well-known chemicals used were sodium hypochlorite or potassium permanganate but unfortunately it led to various disadvantages that gave idea for the use of enzymes. Denim washing mainly includes cellulase enzyme that helps in loosening the indigo dye on the denim called "bio-stonewashing."

18.3.6 Anti-Shrink Treatment for Wool

Traditionally, wool shrinking was achieved by a method called as chlorination. During this process, exo-cuticle of the wool was degraded that form cysteic acid residues and protein losses. Soon this process has been replaced by an enzyme called as proteinases because of its high precision and much lower environmental impact (Uzzal 2013).

18.4 Praiseworthy Effects of Catalyst in Textile Industry

There are several advantages of the utilization of enzyme in the industry. Figure 18.9 illustrates some of the important pros of enzymes.

18.5 Conclusions

Use of enzyme in various processes was developed because they are environmentally friendly and also became a great alternative choice for all the chemical-based processes in approximately all ladders of textile processing. Some commercially thriving applications of amylases, cellulases, and laccases for desizing and denim finishing respectively, and proteases included in detergent formulations are already verified and are well customary. Advance exploration is required for biomodification of synthetic and natural fibers by the accomplishment of profitable enzyme-based processes. In future, broad prospective for innovation and superior enzyme applications needs to be explored in textile processing. Similar to chemicals, these enzymes have their own merits and demerits. The foremost hindrance of using enzymes is that they are expensive. The textile industry is a potent sector where the new methods tailored from biotechnology can be applied, but due to low knowledge it is still not promising. In textile processing there are various steps that effectively add in the use of enzyme like desizing, scouring, and bleaching. The results obtained from the enzymatic processes are same to that of the conventional methods with superior features. With the introduction of the enzymatic processes, several benefits are achieved like trim down the water, power energy expenditure, pollution, and time, and increased quality is achieved. Enzymes (biocatalysts) are

Fig. 18.9 Advantage of using enzyme

rising in a big way in the field of textile industry and if their expense can be managed there utilization in textile industry can be done effectively.

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Role of Enzymes in Textile Processing 19

Ashwini Kumar Dash and Sanat Kumar Sahoo

Abstract

Applications of enzymes to textile wet processing have significantly gained importance due to their stereo specific, non-toxic, eco-friendly, and energy conserving characteristics. The bioprocessing of natural textiles such as cotton, flax, jute, silk and wool has become exceptionally successful in textile wet processing system due to establishment of enhanced functional properties to the fibres along with other essential advantages. Applications to synthetic fibres also proved successful as in case of natural fibres without any exception. Advances in molecular biology and enzyme technology explore the idea of identification of new enzymes and possible alteration of the existing enzymes. In addition, application of enzymes to achieve effective results should also be favourable to technical, economical and environmental concerns. Immobilization of enzymes is one of such approaches for stabilization, providing long life with improved catalytic action to textile substrates. This chapter discusses about different types of enzymes, their potential application areas to different textile material groups, i.e. cellulosic, protein and synthetics, opportunities and challenges associated with these and finally concept of immobilization of enzymes.

Keywords

Enzyme · Natural fibres · Synthetic fibres · Textile wet processing · Bioprocessing · Immobilization

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19.1 Introduction

In today's world, the need of the hour is sustainability with profit making. To have adequate competency in the supply and demand chain, viable technology with quality raw material is highly essential. Varieties of raw materials are available in the market to produce same type of product, but the selection of those raw materials with the appropriate application technology is really a challenging one. Further, to save time as well as to achieve some of the desired properties, selected additives are used during the process of production. These additives are required in order not to compromise with a sustainable as well as quality product. In order to meet the never-ending expectation of humankind, all types of advanced raw materials, chemicals, techniques, high-speed machines, software, etc. are essentially required for a quality outcome. From agriculture to space technology in all sectors, scientists find the way to find a solution in lesser time with best results. In agriculture sector, farmers are in practice to apply pesticides that enable them to gain more crops with less tillage to meet the growing demand. Like this in all sectors, this practice is being followed in order to meet the demand without compromising the quality of the product with the obligation of addressing environmental concerns.

In the field of textiles, there is no exception. The concept of applying enzymes to upgrade the surface properties of fabrics was initially developed in Japan in the year 1989. Gradually, the practice of applying enzymes was increased significantly by many industries in order to achieve desired fabric functional characteristics. From fibre production to finished garments, the use of enzymes has achieved tremendous success from the point of environmental, economical and technological issues. Higher grade of natural and synthetic fibres produced from enzymatic treatment facilitate proper control of the processing parameters like pH, temperature, salt, alkalis, surfactants, etc. during subsequent wet processing stages. Enzymes also help to control the carbon footprint level in the global environment. Moreover, these are naturally available in abundant quantity in the nature as these are produced by living organisms. Enzymes are applied to the fibre producing plants to yield best natural fibres as well as to synthetic fibres by varying the polymeric reaction or modifying the polymeric chain. However, the cost and long-term stability is a matter of concern for the storage and process conditions of these enzymes. New biocatalysts prepared from protein engineering techniques are considered as effective alternatives as these are more thermally stable and perform better even on extreme pH conditions. However, it is not an economical preference for the industries for the mass production in general (Díaz-Rodríguez and Davis 2011; Binod et al. 2013; Singh et al. 2013). This chapter begins with basic concepts of enzymes and their potential applications in all stages of textile wet processing system emphasizing their advantages in comparison to conventional techniques. Later this chapter is focused to the concepts of immobilization of enzymes, which is an approach to improve enzyme properties significantly.

19.2 Enzyme

Enzyme is derived from a Greek word "Enzymos" which means "in the cell" or "from the cell". This is a protein structure of very high molecular weight comprising of over 250 amino acids having distinct active sites within intra-molecular level. These active sites are responsible to execute catalytic reaction. Enzymes as a biocatalyst are very peculiar in their reaction methodology, if compared to inorganic catalysts such as acids, alkalis, metals and metal oxides.

19.2.1 Classification of Enzymes with Specific Functional Properties

International union of pure and applied chemistry (IUPAC) with International union of biochemistry (IUB) established international commission of enzymes (EC) in the year 1956. The commission standardized the terminologies associated with the functional properties of the enzymes in order to facilitate the naming of newly discovered enzymes at that time. These are classified in six broad categories based on their catalytic functions. Each category catalyses the specific reaction as mentioned below.

I. Oxidoreductases: Oxidation/reduction reaction with the substrate.
II. Transferases: Transfer of a functional group between two molecules, e.g. oxidoreductases and hydrolases.
III. Hydrolases: Hydrolysis of various bonds.
IV. Lyases: Cleavage of various bonds other than hydrolysis and oxidation.
V. Isomerases: Changes due to isomerization at intra-molecular level.
VI. Ligases: Involvement in covalent bond formation between two molecules.

Hydrolases and oxidoreductases categories of enzymes are commonly applied to textiles both in preparatory as well as finishing stages of wet processing system.

19.2.2 Advantages of Enzyme Application to Textile Processing

Enzymes lower the activation energy of a reaction by virtue of which the rate of the reaction increases considerably. These act as a catalyst and remain intact until the end of the reaction. These can function at optimum level under mild conditions of temperature, i.e. within 30 °C to 70 °C and pH near to seven. Most of the enzyme activities deteriorate beyond optimum condition limits. These can be used as best substitutes to polluting, carcinogenic, toxic and hazardous chemicals as these are biodegradable and hence safe to environment. Most of the enzymes have high degree of specificity and catalyse the reaction with one or few more substrates. In other words, a particular enzyme catalyses a specific reaction. For example, enzymes opted for desizing do not react with native cellulose hence reduction of strength can be avoided. Thus, the life or wear of garments increases upto a considerable

number of washing cycles. Easily controllable because their activity depends on optimum processing condition. Energy consumption is less as well as non-corrosive in their applications. Lesser chemicals and water are required in various processes, which reduce loads on effluent treatment plants and handling of hazardous chemicals by the workers. The above advantages delineate that enzymes have tremendous potential in the field of textiles for enhancing their ultimate quality in all aspects.

19.2.3 Mechanism of Enzyme Action: Lock and Key Theory

The three-dimensional shape of the active sites available in the enzyme structures is primarily responsible to join with the substrate molecules. Using the concept of lock and key i.e. a right key is necessary for a lock to open, the mode of action of enzyme to its substrate was explained by Fischer in the year 1894. The enzyme is considered as lock and the substrate is the key. This combination enables to form an enzyme-substrate complex in order to facilitate catalytic action. Subsequently, a product is formed by the disintegration of the complex releasing regenerated original enzyme (Zubay et al. 1995) (Fig. 19.1). The active sites of the enzymes are highly specific to their substrates. Right enzymes should fit to right substrates; otherwise, formation of products is unattainable.

Later, the active site of the enzymes becomes inactive and poisoned by a chemical bogie during bioprocessing. This is aided by the intense of temperature, pH and other adverse conditions of the processing environment (Fig. 19.2).

19.2.4 Enzyme Types and Their Specific Application to Textiles

Table 19.1 depicts different enzymes and their specific application to textiles in various processing stage in order to achieve distinct desired fabric properties.

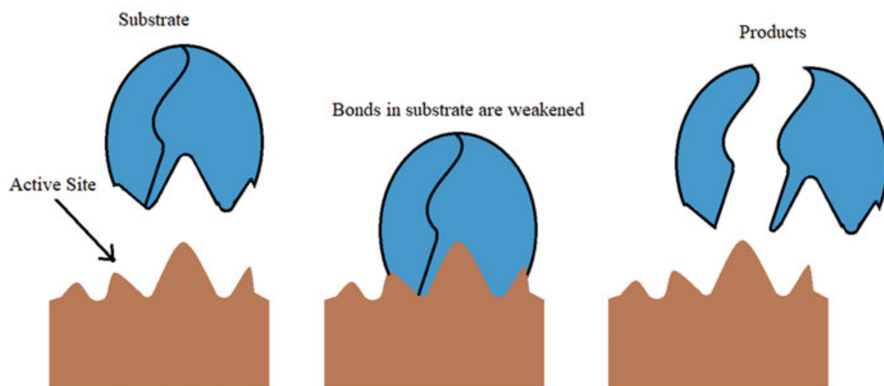

Fig. 19.1 Lock and key model explaining fitting of active sites of enzymes to substrates and subsequent formation of products

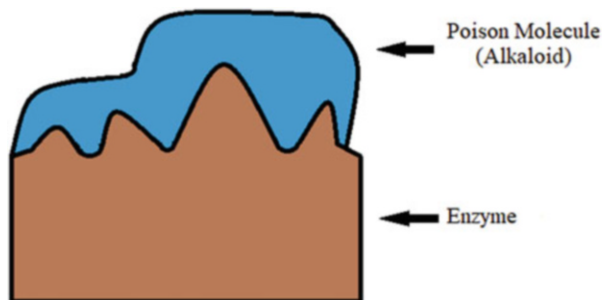

Fig. 19.2 Blocking of active site of the enzyme by poison molecule

Table 19.1 Enzymes for textile usage

Types of enzymes	Usage in textiles and their effects
Cellulases	Biofinishing, biopolishing, pilling and fuzz fibre removal, providing smoothness and softness, improvement in lustre and imparting stone-washed effects on denim
Amylases	To remove starch from the warp yarn, known as desizing
Proteases: (subtilisins)	Inducing shrink resistance properties, removal of impurities and increasing dyeability of wool fibre but with a consequence of weight loss, fabric handle and tear strength
Proteases: (papain)	Degumming of silk but with the possibility of damage to fibroin
Pectinases	Hydrolysis of pectin, e.g. scouring of cotton and wet-retting of flax and hemp fibre
Lipases/Esterases	For increasing hydrophilicity of polyester fibre
Catalases	Neutralize leftover H_2O_2 in the cotton fabric after the bleaching process, hence best for bleach clean-up
Nitrilases and nitrile hydratases	Increase hydrophilicity and dyeability of polyacrylonitrile (PAN) fibres
Laccase	Decolourization of textile effluents and cotton bleaching

19.3 Enzyme Applications in Wet Processing of Cotton

Cotton is established as superior natural fibre for its hydrophilic and comfort characteristics, breathability and durability apart from other exceptional benefits in apparel sector. Abundant literatures are available in the area of application of enzymes to cotton during its wet processing stages. The detailed processing sequences are discussed as follows.

19.3.1 Desizing

Sizing is done to develop a protective coating on the warp yarn surface in order to sustain frictional wear and tear during weaving. Starch is applied to the cotton yarns in general. Starch comprises of a linear as well as a branched polymer known as

amylose and amylopectin respectively. In addition to film forming material, size pastes are prepared with other chemicals which act as binders, lubricants and humectants. Desizing is a process of removal of this coating in order to allow dyes and chemicals to penetrate into the core of the cotton material. Proper desizing helps to avoid uneven dyeing, improper printing and finishing.

Traditionally, rot steeping and the use of acids and oxidizing agents were being practised for desizing. However, the enzymes of amylases types are found to be more suitable and convenient to remove starch-based size as these attack only to starch and does not affect to the native cellulose adversely. For complete hydrolysis, a combination of enzymes such as α-amylase, β-amylase and iso-amylase are essential. α-amylase is a type of endo-acting enzyme, which cleaves the α-1,4-glucosidic linkages in random fashion available both in amylose and in amylopectin starch polymers. This enzyme degrades starch into polymeric fragments of shorter lengths known as dextrins and maltose. These products are disaccharides containing two glucose residues. Beta and iso-amylases are exo-acting enzymes those attack the substrate from the non-reducing end, producing oligo and/or monosaccharides. Excellent biodegradability, avoidance of chemicals, safe to environment and manhandling and the successful reproducibility are the main advantages of enzymatic desizing. Many researchers have concluded enzymes as a superior catalytic performer to cotton textiles from different point of view. Few recent literatures (Agrawal 2016; Shahid et al. 2016; Mojsov 2019) are cited for reference.

19.3.2 Scouring

Scouring is the process of removal of oil, wax, fats and natural colouring matter which are present in the primary wall region of cotton fibre. The basic motto of this process is to cause the fibre absorbent. Noncellulosic contaminants from cotton fibres are removed by sodium hydroxide traditionally. Bioscouring is a process of selective elimination of pectin and waxes from cotton material with alkaline stable pectinase enzymes. This process is very specific to the substrate and does not modify the native cellulose. Further, this can be processed in mild conditions with low consumption of utilities, thus making the process more eco-friendly and energy conserving. Permeableness in the product, no oxy-cellulose formation, minimum loss in strength due to absence of alkali in the bathtub, and low total dissolved solids (TDS) in the discharge wastewater are the added advantages of bioscouring. The major parts of wax are fatty acids, alcohols and esters. These waxes are removed with a high degree of uniformity, which ensures even and homogeneous colouring in subsequent processing stage. This makes it more suitable to blends like viscose, modal, silk, wool, lycra, lyocell, etc. (Wan et al. 2007; Csiszár et al. 2001; Sójka-Ledakowicz et al. 2006). Bioscouring is also more suitable to terry towel and knitted products as softness and fluffiness are more obtainable in comparison to standard scouring.

Pectin, a structural heteropolysaccharide acts as a glue to bind wax and the fibre. The primary chain of it consists of α-1,4-linked D-galacturonic acid. Removal of this

glue definitely eases the removal of non-cellulosic substance from cotton. The pectinase is a type of enzyme that hydrolyses pectin to simpler molecules as galacturonic acids. By means of this hydrolysis, wax becomes extractable or emulsifiable. The pectinase includes pectinesterases, pectin lyases, and polygalacturonases depending on their mode of action (Presa and Tavcer 2007).

19.3.3 Bleaching

Bleaching is an action to remove natural pigments present in cotton fibres in order to provide whitening effect to the fibre. Primarily, bleaching is performed by chlorine and oxygen containing oxidizing agents but they have the following disadvantages. There is a danger of damaging of fabric due to accidental lowering of pH (Basto et al. 2007), the process is slow and carried out at lower temperature and hence difficult for rapid continuous operation, chances of yellowing upon storage and due to relatively high salt loads it is not desirable for environmental safety reason. Gradually H_2O_2 replaced them for their superior bleaching properties but having certain limitations on usage. There is a chance of skin irritation, need of the stabilizer application in order to hold the strength of H_2O_2, decomposition in presence of heavy metals like gold, silver and platinum with the liberation of oxygen, and requirement of high energy to bleach the fabric. Later, many researchers tried bleaching with enzymes using peroxidases, laccase/mediator systems, and glucose-oxidases. But laccase/mediator system bleaching is more specific as these only target to coloured constituents (Nov and Biobeljenja 2013; Špička and Tavčer 2013; Pereira et al. 2005). Enzymatic bleaching certainly gained importance particularly due to low consumption of energy and water, which is most crucial.

19.3.4 Biopolishing

It is a method of treating cellulase enzyme to cellulosic textiles to eliminate micro and fuzzy protruded fibres from the surface. It develops appearance, colour brightness, fabric handle, water absorbency, tendency to less or zero pilling formation and overall surface texture of the fabric. Cellulase derived from the fungus *Trichoderma reesei* is widely used in textile finishing. It contains endoglucanases (EG), cellobiohydrolases (CBH) and β-glucosidases. These act synergistically to hydrolyse the cellulose. Endoglucanases degrade cellulose selectively by attacking the amorphous region of the cotton polymeric system by breaking long polymeric chain to shorter ones. Cellobiohydrolases act sequentially from the ends of glucose chains as a process of degrading cellulose. Cellobiose is thus produced as a major product and plays a mediator role in degrading cellulose. β-glucosidases complete the hydrolysis reaction by converting cellobiose into glucose. A good number of literatures are available in the field of biopolishing of cellulosic textiles with their productive outcomes. Few recent literatures (Moniruzzaman and Reyad 2018; Sankarraj and Nallathambi 2018; Islam et al. 2019; Mojsov et al. 2019) are cited here for reference.

19.3.5 Denim Finishing

Denims are widely used as a casual cotton wear due to their unique faded appearance and durability. Conventionally, these denims are subjected to stone (pumice) washing to accomplish desired aesthetic appeal in addition to other functional properties such as softness and flexibility. However, this stone wash has some inherent disadvantages like difficulties associated with removal of residual stones in the finished garments and overloading of stones that can lead to serious damage to the garments and machineries during the operation. Further, powdered stones formed during the process of denim finishing may lead to clogging of sewerage lines (Yu et al. 2013; Pazarlioğlu et al. 2005).

Cellulase enzymes have the ability to remove indigo dyes captured by the fibres at surface level. These create a fading or frayed look due to the execution of non-homogeneous type of processing. Biowashing with cellulase enzymes is excellent for creamy appeal and superior texture of the fabric. Cellulase enzymes are active and effective in the temperature range of 30° C to 60 °C. Further, they are classified as acid, neutral and alkali within the pH application range of 4.5 to 5.5, 6.6 to 7.0, and 9.0 to 10.0, respectively (Bhat 2000; Sarkar and Etters 2001; Araujo et al. 2008). However, there is an immense possibility of the released indigo dye to come back onto the faded portion of denim; the effect is known as back staining. Works (Araujo et al. 2008; Mojsov 2014) related to this suggested that neutral and endoglucanase rich cellulase preferably works better for softening as well as removal of indigo dyes from the garments. Laccase also can be used as an environmental friendly denim bleaching compared to typical fading process using various chemicals (Rodríguez-Couto 2012).

19.4 Applications of Enzymes in Other Prospective Spaces of Textile Processing

Apart from cotton fibres, protein and synthetic fibres also find tremendous potential to enzymatic treatment for enhanced properties. Some of the prospective application areas are described below.

19.4.1 Anti-Shrink Treatment to Wool

Use of chlorine is a conventional approach to impart shrink resistance property to wool fibre. It has some limitations as the outer layer of this fibre i.e. exo-cuticle degrades, leading to cysteic acid formation resulting in loss of protein. Subsequently, enzymatic treatment of protease was attempted by many researchers (El-Sayed et al. 2002; Cardamone 2002; Ibrahim and Abd-ElSalam 2012) from a different prospective. This showed excellent results such as reducing the adsorbable organic halides (AOX) content in the effluents along with improved whiteness, hydrophilicity and handle characteristics. However, this process also has some disadvantages such as

damaging the fibre cuticle with consequent loss of strength and weight of the fibre (Vílchez et al. 2010; Nolte et al. 1996; Ge et al. 2009; Ibrahim et al. 2012; Silva et al. 2006a, b). Various researchers (Cardamone 2007; Ge et al. 2009; Tesfaw and Assefa 2014) studied that transglutaminase can be used for grafting of amines or proteins in wool fibres in order to achieve favorable results. The immobilization technique can be applied to protease by which the molecular size of the same can be increased. Thus, it imposes restriction to proteolytic attack on the cuticle. The immobilized enzyme continues to reside on the surface layer of the cuticle by hydrolysing the same. By this way, superior tensile strength and optimal felting characteristics of the fibres can be best realized (Schröder et al. 2004; Araújo et al. 2009).

19.4.2 Bioprocessing of Silk

Enzymatic processing reduces the load on the effluents in contrary to chemical processing of silk fibre, which is unquestionably user-friendly and safe to the workers. It produced successful results during preparatory as well as finishing stages of the fibre processing. The different stages of the preparatory process includes cooking of cocoon, degumming, and bleaching. Protease, glucose-oxidase, hexose-oxidase and peroxidase types of enzymes are applied to silk fibre for enhanced properties.

Protease, a hydrolase class of enzymes catalyses the hydrolysis of peptide bonds formed by selective amino acids along with the cleavage of C-N, C-O, C-C bonds. It is successfully applied in the process of cocoon cooking, degumming and biowashing (finishing) stages. Papain protease enzymatic cocoon cooking leads to better silk yield (Gulrajani 2004) along with reduction of labour, energy and environmental pollution. Soap-soda degumming technique is better replaced by alkaline protease enzymes due to controlled action (Gowda et al. 2007) and increased tenacity of the ultimate silk filament. Biowashing and enzymatic finishing using protease impart good handle, pill and shrink resistant properties to the fabric (Arami et al. 2007; Freddi et al. 2003).

Oxidoreductase class of enzymes such as hexose-oxidases and glucose-oxidases catalyse oxidation of maltose and glucose respectively. The liberated H_2O_2 thus obtained due to oxidation, is used for bleaching of protein fibres under mild alkaline conditions to accomplish uniform whiteness in the fabric. However, application of these class of enzymes are not cost effective. Peroxidase, also known as catalases type of enzymes those are applied to neutralize the bleach bath containing surplus H_2O_2. It catalyses the decomposition of H_2O_2 into water and molecular oxygen.

19.4.3 Bleach Clean-Up

Bleaching cotton with H_2O_2 has detrimental effect during dyeing with reactive class of dyes subsequently. The peroxide left with the fabric interferes with the dyes. Hence, an intensive bleach clean-up is essential before dyeing. Traditional method of

neutralization of bleaching with reducing agent or with hot water consumes huge amount of water. Catalases types of enzymes catalyse the breakdown of H_2O_2 into water and molecular oxygen. This is recognized as the best alternative to clean bleach bath before carrying out dyeing with reactive dyes (Gudelj et al. 2001; Amorim et al. 2002). The advantages of bleach clean-up by enzyme include easier and quicker use, less water and energy consumption, less in quantity required, safe to use and less environmental pollution.

19.4.4 Synthetic Fibre Modification to Enhance Functional Properties

Enzyme applications improve many functional characteristics of synthetic fibres such as hydrophilicity, weavability, resistance to pilling, affinity to dyeing, finishing complications and resistance to static charge generation. Enzymes find better application to polymer synthesis, improve surface properties of fabrics as well as polymer grafting of textile substrates.

19.4.4.1 Bioprocessing of Polyester and Their Characteristics

Hydrolases class of enzymes such as lipases, cutinases and esterases are best suitable for modification of polyester fibre. Among these three, esterases are less effective to hydrolyse polyester at surface level. However, new esterases from *Thermobifida halotolerans* are found to be best for surface hydrolysis of both PET and PLA (Ribitsch et al. 2012). Lipases from *Triticum aestivum, Burkholderia* spp., *Humicola* sp., *Candida Antarctica*, *Rhizopus delemar, and Thermomyces lanuginosus* perform better to hydrolyse polyester (Gübitz and Paulo 2003; Walter et al. 1995). Cutinases, particularly representatives from *Aspergillus oryzae, Penicillium citrinum, Fusarium solani, Thermobifida fusca, F. oxysporum, Thermobifida cellulosilytica and Humicola insolens* also have been found very much effective to hydrolyse polyester (Silva and Cavaco-Paulo 2008; Gübitz and Paulo 2003; Kanelli et al. 2015). Mixer of cutinase and lipase was also tried to investigate the impact on hydrolysis of polyester (Lee and Song 2010). Hydrolysis of polyester using oxidative enzymes such as laccases showed good results without polymer cleavage (Miettinen-Oinonen et al. 2002). Digital printing of polyester fabric using disperse inks showed substantial improvement in colour fastness properties (Ibrahim and Abd-ElSalam 2012).

19.4.4.2 Bioprocessing of Polyamide and Their Characteristics

Amidases, proteases, peroxidases and cutinases types of enzymes exhibited successful results to improve functional characteristics of polyamide fibres. Laccases substantially increased the hydrophilicity of nylon 6,6 fabrics when acted with a mediator (Silva and Cavaco-Paulo 2008; Miettinen-Oinonen et al. 2002). An amidase from *Nocardia* sp., a cutinase from *F.solani pisi* and protease from *Beauveria* sp., when treated with nylon 6,6 fabrics showed good absorbency to reactive and acid dyes (Parvinzadeh 2009). Protease (Parvinzadeh 2009) and lipase (Kiumarsi and Parvinzadeh 2010) treated nylon 6 fabrics offered higher exhaustion rate to acid

and disperse dyes. It was confirmed that cationic dye affinity (El-Bendary et al. 2012) and hydrophilicity (Begum et al. 2016) of nylon fabric are improved by protease from a novel *Bacillus isolate* without any hamper to the mechanical properties of the fabric. The concentration of lipase enzyme was found to be directly proportional to the hue of the dyed samples (Gashti et al. 2013). Treatment on wool/nylon blended fabric with protease enzyme was also carried out to explore changes in morphological structures, wettability and dyeability (Waly et al. 2016; Parvinzadeh 2007). Nitrilases or any other enzymes comprising of nitrile hydratase and amidase, hydrolyse the nitrile groups of polyacrylonitrile (PAN) to the corresponding acids or amides, respectively. Thus, there is a chance of considerable increase in hydrophobicity of PAN.

19.4.5 Biological Polymer Synthesis and Functionalization

Researches are being stressed upon biocatalytic approach on polymer synthesis and functionalization due to the toxicity associated with metal catalyst residues and solvents. Better polymer functionalization can be obtained with precise modification of polymer structure by some selective enzymes (Sen and Puskas 2015; Miletić et al. 2012; Gübitz and Paulo 2003).

19.4.6 Immobilization of Enzymes for Improving Textile Functional Properties

Immobilization of enzymes is a technique to reduce or restrict the movement of the same with the retention of their catalytic activity. It has also the ability of preserving the capability to continuous and repeated usage. This is achieved by attaching the enzymes with support or carrier materials (Brena et al. 2013; Tischer and Wedekind 1999; Khan and Alzohairy 2010). Immobilization of enzymes is established as the best industrial bioprocessing method particularly for food and pharmaceutical industries. This technique also turned out to produce significant results to textiles as well.

Hydrolase class of enzymes are superior for imparting antimicrobial properties to textiles. By means of hydrolysis of cell wall polysaccharides, these enzymes act as an obstacle to the intrusion of microbes. Lysozymes are another kind of enzymes, which proved to be successful against many pathogenic bacteria. Further, their immobilization has potential outcome to create smart materials with the property of self-detoxification and antimicrobial attributes (Paul and Genescà 2013; Shen 2019). Antimicrobial property of some textiles can also be improved upto an extent of minimum ten washing cycles by the attachment of alkaline pectinase, α-amylase or laccase on them (Ibrahim et al. 2007). Specifically in case of wool fibres, this have been reported as successful with immobilized lysozyme using microbial transglutaminase (Huang et al. 2009a, b) and glutaraldehyde (Hu et al. 2008; Wang et al. 2009) as cross-linkers. Lysozyme when covalently bonded with wool

fibre showed antimicrobial properties with good durability upto a certain number of laundering cycles (Shah and Halacheva 2016). Cross-linking of microbial transglutaminase with lactoferrin showed significant antimicrobial properties of wool fabrics as a process of immobilization against *E. coli and S. aureus* compared to native lactoferrin (Han et al. 2014). Lauryl gallate, a phenolic water insoluble compound was successfully grafted on wool fibres by laccase enzymes to accomplish antioxidant, antimicrobial and hydrophobic properties of the fibre. Further, grafting of nordihydroguaiaretic acid (NDGA) on wool fibre was done successfully by laccase to enhance functional properties such as UV protection, tensile strength and shrink resistance characteristics of the fibre. Kinases are used as a process of enzymatic phosphorylation to impart flame retardant properties to natural textiles. Several literatures are cited regarding the use of this immobilization technique in the textile processing system to achieve exceptional benefits from economical, technological and environmental aspects. Few recent literatures (Besegatto et al. 2018; Coradi et al. 2018; Sankarraj and Nallathambi 2018; Darwesh et al. 2019; Morshed et al. 2019) are cited here for reference.

19.5 Prospects and Challenges Associated with Applications of Enzymes

Immobilized enzymes restrict surface hydrolysis of cotton fibre during denim fading, biopolishing and anti-shrinking treatment of wool fibres. However, limited researches have been carried out on application of immobilized enzymes to synthetic fibres. Immobilized enzymes have promising prospects in the field of discolouration of textile effluents (Rauf and Ashraf 2012; Khan et al. 2013). Laccase can also function effectively in the chemical processing of cotton textiles in various ways like increasing fastness properties and in situ formation of coloured pigments (Kim et al. 2007; Hadzhiyska et al. 2006; Couto and Herrera 2006). Alkaline catalase was successfully applied to develop sulphide native redox systems to facilitate reduction of sulphur dyes for successful dyeing of cellulosic textiles (Chakraborty and Jaruhar 2014).

However, properties of enzymes change very often due to immobilization. In addition, the size of enzymes also increases leading to the development of insolubility characteristics. This leads to poor interaction between the enzymes and insoluble substrates, thereby limiting the diffusion of the former inside the latter. Destabilization of enzymes cannot be avoided due to the existence of various chemicals and auxiliaries in textile materials. Use of nanoparticles and smart polymers as a support for the application of enzymes in textiles were cited by many researchers (Soares et al. 2011; Yu et al. 2013; Brena et al. 2013). Enzymes linked with smart polymers or nano-sized materials increase diffusion rate to the substrates along with increasing stability of the system as a whole. This research is still continuing to meet the challenges associated with mass transfer constraints of the immobilized enzymes.

19.6 Conclusions

Application of enzymes is a prospective area in the field of textile wet processing. These are supposed to be the best alternative to chemicals as this process works underneath delicate operating conditions of temperature and pH, thereby reducing energy consumption and load on effluents. Application of enzymes in colouring is another potential area although success in commercial scale is yet to be achieved. There is enough scope for any add-on coloring with varied dye–fibre systems. Immobilization of enzymes and their application to textiles is one of the most promising and competent technique from technical, economical and environmental point of view. Due to wide variation of properties of individual enzymes and complications associated with their reaction mechanisms, there is no straightforward procedure or standard for support and method of immobilization. The ultimate choice of immobilization procedures as well as carrier materials depends on specific application.

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