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Pradeep Verma *Editor*

Thermochemical and Catalytic Conversion Technologies for Future Biorefineries

Volume 2

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Series Editors

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The consumption of fossil fuels has been continuously increasing around the globe and simultaneously becoming the primary cause of global warming as well as environmental pollution. Due to limited life span of fossil fuels and limited alternate energy options, energy crises is important concern faced by the world. Amidst these complex environmental and economic scenarios, renewable energy alternates such as biodiesel, hydrogen, wind, solar and bioenergy sources, which can produce energy with zero carbon residue are emerging as excellent clean energy source. For maximizing the efficiency and productivity of clean fuels via green & renewable methods, it's crucial to understand the configuration, sustainability and techno-economic feasibility of these promising energy alternates. The book series presents a comprehensive coverage combining the domains of exploring clean sources of energy and ensuring its production in an economical as well as ecologically feasible fashion. Series involves renowned experts and academicians as volume-editors and authors, from all the regions of the world. Series brings forth latest research, approaches and perspectives on clean energy production from both developed and developing parts of world under one umbrella. It is curated and developed by authoritative institutions and experts to serves global readership on this theme.

Pradeep Verma
Editor

Thermochemical and Catalytic Conversion Technologies for Future Biorefineries

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Dedicated to my beloved mother.

Preface

There is a need to explore alternatives to fast depleting fossil fuels in order to meet the global rise in energy needs due to increasing population and modernization. Waste biomass (such as agricultural waste, forest waste) and algal biomass are considered to be one such alternative. Apart from this waste biomass, hybrid energy crops can also be converted to biofuel and different value-added chemicals. Therefore, different biomass-based biorefineries such as lignocellulosic, algal-based, and food waste-based biorefineries are developed which are later aimed to be integrated biorefineries similar to conventional petroleum refineries. However, the utilization of these biomasses for efficient high-value product generation is a multistep process involving pretreatment, hydrolysis, fermentation, recovery, etc. The pretreatment and hydrolysis of these biomasses are essentially required to break down complex natural polymers into simple compounds that can be used for further conversion to the fuel of high-value compounds. The pretreatment and hydrolysis are also considered crucial stages of this biomass-based biorefinery and involve several thermochemical and advanced catalytic technologies. This book is an attempt to provide an account of biomass recalcitrance and available physical and chemical methods for biomass pretreatment and hydrolysis. It focuses on understanding the critical role of enzymes in the development of integrated biorefinery. The book also presents an overview of the utilization of waste biomass as a support system for enzyme immobilization for easy recovery and reuse for multiple cycles, strategies where enzymes can be used. The book also attempts to understand how enzymes can play a vital role in waste valorization for energy and biomaterial production. Further, the book will present an overview of how advanced technologies such as omics and in silico approaches can help in understanding the chemistry affecting recalcitrance and the mechanism of enzyme catalysts in their bioconversion. An understanding of the life cycle assessment of waste biomass biorefinery will be needed before its

implementation. The book will serve as an additional reading material for undergraduate and graduate students of energy studies, chemical engineering, applied biotechnology, and environmental sciences. This book is of interest to academicians, scientists, environmentalists, and policymakers.

Ajmer, Rajasthan, India Pradeep Verma

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I am always thankful to God and my parents for their blessings. I also express my deep sense of gratitude to my wife Savita and my son Mohak and daughter Netra for their support during the development of the book and in life.

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Pradeep Verma is a Professor at the Department of Microbiology, School of Life Sciences, CURAJ, Ajmer, India. He worked as an Associate Professor and Reader-Founder Head at GGV, Bilaspur and Assam University, Silchar. He received his Ph.D. from Sardar Patel University, Gujarat, and his Master's and Bachelor's from MDS University, Rajasthan. He worked as visiting scientist at UFZ Centre for Environmental Research, Halle, Germany, and a Post-doctoral Fellow at ASCR Prague, Gottingen University, Germany, and RISH Kyoto University, Japan. He also worked at Reliance Life Sciences, Mumbai, India. His stay at Japan and Reliance Life Sciences resulted in several commercialized National and International patents. He is a recipient of various prestigious awards and fellowships such as the Ron Cockcroft Award by Swedish Society, UNESCO Fellow, DFG Fellow, and JSPS PDF. He is a fellow of the Mycological Society of India and the Biotech Research Society of India. His area of expertise involves microbial diversity, bioremediation, bioprocess development, lignocellulosic, and algal biomass-based biorefinery. He has contributed to 71+ research papers, 40+ book chapters, and 11 edited books. He is an editorial board member and reviewer of several prestigious high-impact journals.

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Chapter 1
Lignocellulosic Biomass Valorization and Fate of Recalcitrant

Atul Srivastava, Tanmay Rohit, Meenakshi Singh, Yaseera Bhombal, Sanjeet Mehariya, Obulisamy Parthiba Karthikeyan, K. Chandrasekhar, and Murthy Chavali

Abstract Valorization of lignocellulosic biomass (LCB) as a promising alternative to bioenergy and value-added products and for biorefinery application has fetched a lot of attention in the past few decades. LCB comprised of cellulose, hemicelluloses, and lignin, which converse a recalcitrant structure, that makes it inefficient for valorization through a biological approach. This review aims to emphasize the fate of recalcitrant LCB and understand their fate during biomass utilization. In specific, LCB recalcitrance for valorization is compared by various physio-chemical pretreatment techniques, and their combinations with enzyme treatment are discussed. It was found that the combined treatments are more effective, while their effects on recalcitrant removal were totally dependent on structural and

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functional factors of LCB. Therefore, a detailed investigation of efficient LCB conversion protocol into viable bioproducts needs more research.

Keywords Lignocellulosic biomass (LCB) · Recalcitrance · Enzymes · Value-added products · Biorefinery · pretreatment

Abbreviations

AFEX	Ammonia fiber expansion
ASA	Accessible surface area
DP	Degree of polymerization
DSAP	Dilute sulfuric acid pretreatment
LCB	Lignocellulosic biomass
LHW	Liquid hot water
LPMOs	Lytic polysaccharide monooxygenases
SCP	Sodium chlorite pretreatment

1.1 Introduction

The overexploitation of natural assets has constrained the fast-developing populace and its steadily expanding energy interest to seek sustainable inexhaustible assets to a quick transition from non-renewable energies to lignocellulosic biomass (LCB). LCB is primarily made of cellulose and hemicelluloses and an aromatic polymer called lignin. Lignin is mainly comprised of phenolic and non-phenolic compounds (Sarkar et al. 2009). Lignin gives mechanical stability to the secondary cell walls of vascular plants, thus increasing tolerance towards various biotic and abiotic stress and enhancing the energy value in biomass conversion (Frei 2013). If the forage is rich in lignin content, it is not digestible by the rumen microbiota in the animals and therefore recalcitrant. Moreover, few bacteria, fungi, and insects can produce lignin-digesting enzymes and reduce the LCB recalcitrance (Zoghlami and Paës 2019; Agrawal and Verma 2020, 2021; Agrawal et al. 2021; Bhardwaj and Verma 2020).

LCB is the most abundant biomass on the planet and is usually used to produce value-added products like ethanol, acid, saccharides, phenols, aldehydes, xylitol, and cellulose acetate by various processing treatments without any negative environmental impact (Ribeiro et al. 2018; Kumar and Verma 2020a). Therefore, LCB is considered a suitable feedstock for alternative fuels, and chemical and material products by fermentation. However, the conversion process results in the recalcitrance of LCB because of the complexity and heterogeneity of the biomass structure (Rajesh Banu et al. 2021; Kumar et al. 2020). Many recalcitrant factors like molecular weight and chemical structure of lignin, crystallinity, and polymerization degree of cellulose are responsible for the biological transition of biomass that

prevents the enzymatic hydrolysis of cellulose (Li et al. 2016). Further, some interning linkages of lignin structure specifically hydroxyl, phenolic, aliphatic, and other aromatic subunits impact the recalcitrance of plant biomass to prepare bioproducts (Zoghlami and Paës 2019). Because of these recalcitrant factors, it has been extensively documented that lignin is susceptible to biomass conversion processes and requires chemical pretreatment to avoid condensation on the secondary walls by enzymatic hydrolysis (Ubando et al. 2021). But still, there is uncertainty about the exact mechanism of enzymatic breakdown of LCB which still needs more investigation studies by examining different operating conditions and pretreatment regimes in LCB valorization. Because lignin–enzyme interactions are crucial for the understanding of the cell wall recalcitrance measured by biomass enzymatic hydrolysis. It depends on limiting polysaccharides availability which also inhibits binding capacity with enzymes (Carpita and McCann 2020; McCann and Carpita 2015).

The overexploitation of petroleum for fuel and increasing industrialization have caused a drastic reduction in petroleum availability and look for alternative resources to meet the future supply and demands. LCB is the largest natural renewable carbon source for biofuel production like bioethanol, biofuel, and biohydrogen. But the recalcitrant nature of the lignin is the major challenge in biofuel production because, in the presence of lignin, the cellulose is not accessible to enzyme cellulase for the hydrolysis of the cellulose for further conversion. Therefore, pretreatment is one of the most important steps in biofuel production (Sivagurunathan et al. 2017; Bhardwaj et al. 2020). Table 1.1 list out some important research and reviews published on LCB and recalcitrance of different plant biomass. This chapter aims at current state-of-the-art findings on the LCB treatment for biomass utilization involving different pretreatment regimes such as milling, microwave-assisted alkali treatment, pyrolysis, ultrasonication, organosolv, ammonia fiber expansion, ionic solvolysis, fungal enzymatic degradation, and others to decrease LCB recalcitrance. It also covers various structural and chemical factors affecting LCB recalcitrance which are kept in mind to work for strategic developments in creating an efficient LCB conversion protocol into viable bioproducts. Specifically, downstream processing of lignin condensation and repolymerization can be focused to modify the structural component of lignin to avoid any undesirable chemical reactions using capping agents.

1.2 Plant Cell Characterization for LCB

Lignocellulosic biomass (LCB) is a plant-based complex matrix made up of different polymers like lignin $[C_9H_{10}O_3(OCH_3)0.9–1.7]x$, cellulose $(C_6H_{10}O_5)n$, and hemicellulose $(C_5H_8O_4)m$ with minute quantities of protein, ash, and pectin mixed in it (Østby et al. 2020). Figure 1.1 shows the LCB composition chiefly cellulose (33–51%), hemicelluloses (19–34%), and lignin (20–30%) (Trajano et al. 2013). LCB has a spatial structural arrangement wherein cellulose is covered by hemicelluloses and lignin. Cellulose and hemicelluloses form a part of the primary cell

Table 1.1 Research conducted on LCB recalcitrance using different plant biomass

Type of plant biomass	Findings	References
Lignocellulosic matrix	Pretreatment by mechanical activation of metal salts (chlorides and nitrates) increases the hydrophilicity of enzymatic conversion in LCB without removing or weakening the lignin content	Zhang et al. (2019)
Lignocellulose substrates (birch, pine, walnut shell, and reed)	The two-way synergy between enzymatic treatment and ball milling efficiency to isolate lignin with high yield, high native linkage content, purity, and minimal molecular weight reduction	Wang et al. (2021)
Chinese fir sawdust	Effects of dilute sulfuric acid pretreatment (DSAP), acidic sodium chlorite pretreatment (SCP), and their combined pretreatments (DSA-SCP and SC-DSAP) on Chinese fir sawdust. The outcome reveals that the order of sequential pretreatment significantly affected the delignification, and hemicellulose should be removed first	Ouyang et al. (2021)
Glucan and xylan	Superior residence times of the lignocellulosic biomass under mild conditions (120 °C) can increase the glucan and xylan hydrolysis due to effective hemicellulose depolymerization	Hong et al. (2016)
Hemicelluloses and acetyl groups	Removal of hemicelluloses on pretreated pine improved the fibers porosity and the area available for enzymes. It has also been reported that removal of hemicelluloses by dilute acid or steam explosion pretreatment could increase cellulose conversion by improving the accessibility of enzymes to cellulose	Zoghlami and Paës (2019)
Fiber, fibril, and microfibril	Microaerobic pretreatment is an efficient and cost-effective pretreatment method that meets most of the requirements for industrial applications, such as the formation of reactive cellulosic fiber for the enzymatic attack, the avoidance of the formation of possible inhibitors to the fermenting microorganisms and hydrolytic enzymes, reduced energy demand, and reduced cost of size reduction of the feedstock	Amin et al. (2017)
Plant biomass	Pretreatment and saccharification (SPS) using a cocktail of hydrolytic and oxidizing enzymes from the fungal consortium. This is the first report on the development of an eco-friendly simultaneous pretreatment and saccharification methodology. This process completely eliminates the use of hazardous chemicals. Conducting pretreatment and saccharification in the same vessel makes the process economically viable, reduces energy consumption, and generates a simple process for the removal of residual biomass	Dhiman et al. (2015)

(continued)

Table 1.1 (continued)

Type of plant biomass	Findings	References
Corn stover and wheat straw	They found that the influences of pretreatments also differed depending on the biomass substrates. Hydrothermal, dilute acid pretreatments increased the relative content of lignin in the solid residues because of the significant removal of hemicellulose with minimal loss of cellulose and lignin	Jensen et al. (2017)
Cellulose and hemicellulose	Copper-dependent enzymes, lytic polysaccharide monooxygenases (LPMOs), have changed the view of employing hydrolytic enzymes only for the degradation of cellulose and hemicellulose. LPMOs can increase efficiency and reduce the cost of fermentation.	Haq et al. (2021)
Switchgrass, corn, Miscanthus	To reduce the pretreatment time, reported the use of microwave irradiation (800 W). They observed a significant drop-in pretreatment time (45 s) during the pretreatment of switchgrass, corn stover, and Miscanthus, using ChCl: lactic acid (1:2) DES. The CrI for switchgrass, corn stover, and Miscanthus also increased as compared to that of the untreated feedstocks	Chen and Wan (2018)

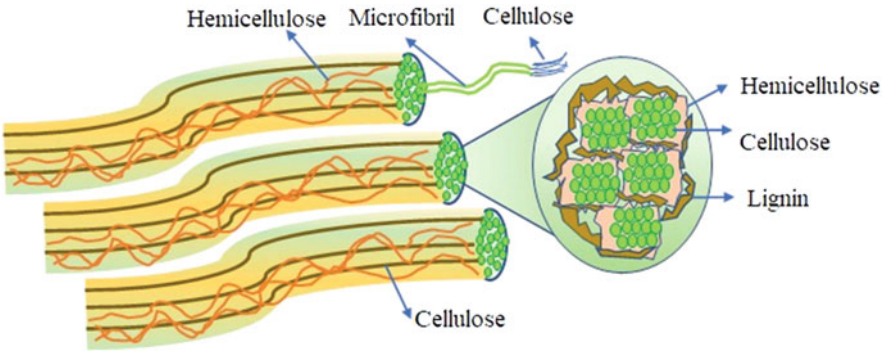

Fig. 1.1 Schematic illustration of the structures and the major biopolymers in plant cell walls (Zhao et al. 2019)

wall, while lignin along with other constituents forms the secondary cell wall of plants. The composition of LCB depends upon the requirement of cellular function, mechanical properties, and permeability.

1.2.1 Lignin

In LCB, lignin is the main non-carbohydrate, racemic, cross-linked, and heterogeneous biopolymer. It is chemically stable and heat resistant as compared to cellulose. The arrangement of monolignols p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol creates a network of hydroxycinnamyl monomers, p-hydroxyphenyl(H), syringyl (S), and guaiacyl (G) (Vanholme et al. 2010). These hydroxycinnamyl monomers are methoxylated to varying degrees (Li et al. 2016). Lignin is incorporated between hemicelluloses and cellulose to cohere them together. The lignin content helps in providing structural rigidity and protects polysaccharides from degradation by microbes (Ni et al. 2020). It is responsible for hydrophobicity in the cell wall which in turn helps inefficient water conduction in vascular plants. Lignin has been reported as a powerful recalcitrant agent affecting biomass recalcitrance (Meng et al. 2017; Kumar and Verma 2020b; Bhardwaj and Verma 2021). The amount of lignin, its monomer composition, and distribution play a crucial role in biomass recalcitrance. This effect of recalcitrance by lignin is caused by both physical and chemical properties. Physical factor involves limiting enzymatic hydrolysis by reducing the availability of cellulose to the enzyme. This is achieved by covering the cellulose fibrils. Lignin has hydrophobic structural features which serve as a chemical factor for recalcitrance. The hydrophobic groups in lignin adsorb the enzymes involved in hydrolysis irreversibly, thereby preventing hydrolysis of cellulose (Kumar and Wyman 2009; Yao et al. 2018; Bhardwaj et al. 2021). Hence, pretreatment of lignin becomes an imperative phase in biofuels generation via LCB to gain access to cellulose polymer.

1.2.2 Cellulose

Cellulose is a linear polysaccharide made up of β(1–4) D-glucopyranose units, which are repeated inversely at every alternative position forming cellobiose units linked by β(1–4)linkage. Each chain is made up of approximately 500–1400 D-glucose units to make microfibrils that again aggregate to develop cellulose fibrils (Robak and Balcerek 2018). In nature, cellulose is found in both crystalline and amorphous morphologies in terrestrial plants. Though cellulose is insoluble in the water and acts as a scaffolding framework that provides tensile strength to the cell. But this strength depends upon the degree of polymerization. The longer the chains more difficult it becomes to hydrolyze the chain due to the higher number of hydrogen bonds. This suggests that hydrogen bonds facilitate enzyme accessibility toward cellulose (Meng et al. 2017).

1.2.3 Hemicellulose

It is a heteropolymer of five or six carboned sugar monomers. It has a β(1–4)linked backbone with all the monomers in equatorial configuration at C1 and C4 positions (Scheller and Ulvskov 2010). Hemicellulose is amorphous, possesses a low tensile strength, and is highly soluble in diluted acids or bases because of hemicellulosic enzymes (Isikgor and Becer 2015). It is positioned tethered alongside cellulose. It is a short-chained branched polysaccharide consisting of around 500–3000 sugar residues. These sugar residues mainly include glucose and xylose and may also include galactose, rhamnose, mannose, arabinose, mannuronic acid, and galacturonic acid. The degree of polymerization is in the range of 100–200 units based on the monosaccharide repeating units, hemicelluloses are classified into four types: xylan, mannan, (1:3, 1:4), β-glucan, and xyloglucan. Xylan consists of the monomers xylose, rhamnose, and galacturonic acid, mannan of β(1–4) mannose, (1:3, 1:4) β-glucan of D-glucose, and xyloglucan made up of β(1–4) linked D-glucose substituted by xylose (Scheller and Ulvskov 2010).

The hemicelluloses create a cross-linked network and provide structural integrity to the cell. Different plant species show different monomeric subunits in their hemicellulose polymers (Zhang et al. 2016). Hemicellulose also acts as a barrier limiting the availability of cellulose for enzymatic hydrolysis. Hemicellulose in LCB is sometimes found to have acetylated groups. These acetyl groups play a key role in providing recalcitrance. They interfere with enzyme recognition and also hinder the binding of catalytic domains of cellulases to the substrate by changing its hydrophobicity (Pan et al. 2006).

1.3 Factors Affecting LCB Recalcitrance

The enzymatic hydrolysis of lignocellulose is affected by several factors. The two main categories of lignocellulose based on structural composition are physical structure and chemical and those relating to the cellulolytic enzyme processes and interactions (Fig. 1.2). However, the structural properties of lignocellulose prove to be the key limiting factor in lignocellulose enzymatic hydrolysis. There have been identified several structural factors affecting the digestibility of biomass enzymes: lignin content, hemicelluloses, and cell wall proteins, degree polymerization level (DP), cellulose crystallinity, particulate size, accessible surface, the thickness of cell walls, and pore depth (Zoghlami and Paës 2019).

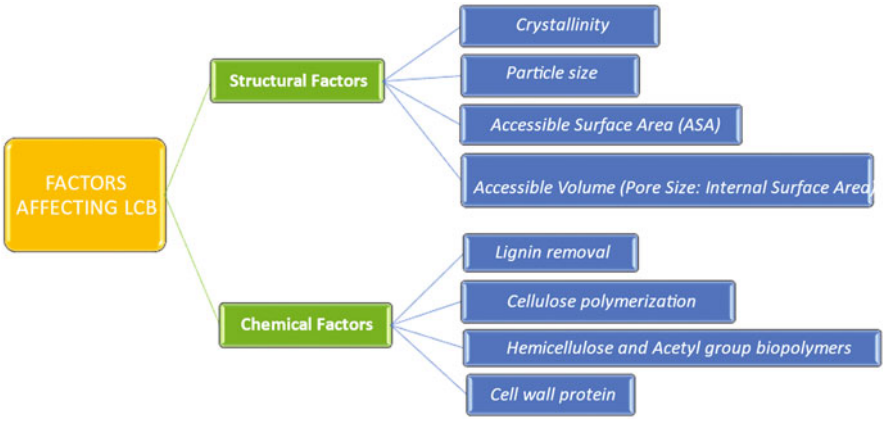

Fig. 1.2 Factors affecting LCB recalcitrance undergoing enzymatic interactions

1.3.1 Structural Factors

1.3.1.1 Crystallinity

The crystallinity of cellulose, a supramolecular property, is one of the most detailed researched subjects. The ratio of crystalline and amorphous regions is represented by non-covalent hydrogen bonds making their enzymatic hydrolysis around 3–30 times smaller than that in the amorphous regions strongly interrelating the crystalline cellulose fibers (Guo et al. 2014). However, crystallinity has various effects on hydrolysis. Some studies have shown that the enzyme has a negative relationship to the enzymatic hydrolysis, especially at the initial hydrolysis rate on pretreated wheat straws (Pihlajaniemi et al. 2016) and on pre-treated maize stover. Others showed that hydrolysis is less important than other physical properties such as DP, pores length, accessibility to the surface, and particle size to reduce crystallinity (Mansfield et al. 1999). The crystalline structure has proven that the heterogenity of the LB and can be used to calculate the saccharification yield.

1.3.1.2 Particle Size

Particle size is a primary parameter influencing the ability of cellulose hydrolysis. The removal of particulate matter by framing, grinding, and extrusion may enhance enzyme–cellulose relationships and decrease lignocellulose compact structure and hence increase hydrolyze rate (da Silva Coelho-Moreira et al. 2013). Studies have shown that enzyme hydrolysis is enabled by the mechanical deconstruction of different feeding stocks, e.g., wood chips. Jiang et al. (2017) noted that a decrease in particle size below 400 μm would adversely impact poplar hydrolysis as compared to the size threshold of wheat straw was 270 μm.

1.3.1.3 Accessible Surface Area (ASA)

The available surface area (ASA) of LCB is a crucial enzyme hydrolyze element, closely related to the structural characteristics of the porosity like pore depth and unique surface (SSA) (Liu et al. 2019). An increase in ASA is observed when there is an increase or decrease in the pore volume in particle size. The enzymatic conversion of pretreated pine wood is improved by ASA and has also been stated that ASA can help the hydrolytic enzymes by improving its access of fiber, for aspen wood. ASA is hard to predict, however, SSA is also considered to calculate the actual enzyme surface (da Silva Coelho-Moreira et al. 2013). In addition, the smaller is the element, so greater is the SSA recorded by the hydrothermal pretreated maize stover, with a 138% increase in enzyme digestibility (Karimi and Taherzadeh 2016).

1.3.1.4 Accessible Volume (Pore Size: Internal Surface Area)

The accessible cellulose volume in LCB is an essential factor in enzyme deconstruction (Jeoh et al. 2007). According to Grethlein (1985) "pore volumes are more or less available to enzymes, depending on their size or form. The cellulase size is usually around 5.1 nm and so enzymes can only be accessible by pores greater than 5.1 nm." The enzymatic conversion and pore size of biomass yields for dilute acid pretreated poplar are closely linked to cellulosic substrates (Meng et al. 2015). The biomass-specific and pretreatment-dependent relations with specific porosity vary, for example, the hydrolysis production was closely related for the wheat straw to pores of 15–30 nm, whereas it correlated for poplar to pores of 10–15 nm. Only pores below 10 nm have a good hydrolysis connection in *Miscanthus*, which has been demonstrated to suggest that there are no specific pores that increase the hydrolysis yield (Herbaut et al. 2018). A study by Ishizawa et al. (2007) found a lack of association between hydrolysis yield and pore size for the dilute maize stover pretreated acid pine pretreatment and dilute acid pretreated and delignified sugarcane, respectively. Furthermore, the rise in pore volume when the lignin content does not exceed 15% shows that the enzyme digestion of pines pretreated has a neglected impact (Santos et al. 2012).

1.3.2 Chemical Factors

1.3.2.1 Lignin Removal

After cellulose, lignin is the second-largest polymer in LCB, which constitutes a dry weight of around 15–40% (Sannigrahi et al. 2010). The amorphous phenylpropanoid building element heteropolymer is very complex (sinapyl alcohol, P-coumaryl, and coniferyl). Many scientific findings have confirmed that lignin reduction improves the digestibility of cellulose (Chang and Holtzapple 2000). This is perhaps the most

important procedure in alkaline precursors to enhance cellulose digestibility efficiently without full hemicellulose removal. Lignin primarily restricts biomass enzyme hydrolysis from two dimensions.

The removal of lignin usually disrupts and increases porosity and reduces non-productive lignin carbohydrate enzyme adsorption (Pihlajaniemi et al. 2016). Phenolic hydroxyl groups (line-dependent compounds) have been reported to be responsible for reversible cellulase inhibition (Lv et al. 2013). The inhibitory activity of lignin decreases considerably (by 65–91%) by the chemical reactions of free phenolic hydroxyl groups, like hydroxypropylation reaction. The ratio of S-G lignin is an independent factor in recalcitrance (Yoo et al. 2020). Regardless of this, earlier research has found little impact on enzyme hydrolysis in the S/G ratio of untreated LB: untreated S/G poplar ratio between 1.0 and 3.0, G-rich and S-rich lignin stems (Li et al. 2010), and transgenic alfalfa (*Arabidopsis*) (Chen and Dixon 2007). Thus, LB recalcitrance is greatly caused by lignin, which gets affected due to its structure and chemical composition.

1.3.2.2 Cellulose Polymerization

The degree of cellulose polymerization (DP) denotes the amount of glucose units in the polymer that has a significant influence on LB recalcitrance. However, its specific purpose is not obvious yet, and the present amount of information is impossible to analyze individually (Kumar and Sharma 2017; Verma and Shah 2022). Changes in structural characteristics such as porosity and crystallinity are always followed by changes in DP (Yoo et al. 2020). An experiment on α-irradiation that caused cotton linters to decrease their DP only had a modest influence on the saccharification rate and is reported by (Sannigrahi et al. 2010).

1.3.2.3 Hemicellulose and Acetyl Group Biopolymers

Hemicellulose and acetyl group biopolymers account for 20–35% of the weight of heterogenous biomass (Chandel et al. 2018). It includes several subsets of monosaccharide forming mannans, xylans, glucomannans, xyloglucans, and other products. The value of the degree of polymerization of hemicelluloses lies between 100 and 200 units, which is lesser than that of cellulose (Rodrigues Mota et al. 2018). Although it may have complexity more or less by higher substitution level. Hemicellulose has a poor muscular intensity and is amorphous. Diluted acids or bases and hemicellulase enzymes are readily hydrolyzed (Isikgor and Becer 2015). The enzyme's availability is being restricted by hemicelluloses which cause physical obstruction. The removal of hemicellulose using dilute acid or steam explosion pretreatment has been reported to boost the conversion of cellulose by improving cellulose enzyme access (Auxenfans et al. 2017) documented hemicellulose reduction of pretreated pine increased porosity of fibers and enzyme range.

Some experiments have found that the removal of hemicelluloses is more effective than the removal of lignin to increase the enzymatic rate of hydrolysis (Leu and Zhu 2013). The formation of productive binding between the catalytic domain of cellulases and cellulose might get hindered by changing cellulose's hydrophobicity or by increasing the diameter of the cellulose chain (Pang et al. 2019). Past research on corn stover reported that enzyme effectiveness can be improved by reducing the acetyl content (Kumar and Wyman 2009). However, other studies on switchgrass, bagasse, wheat straw, poplar wood, etc., have shown that the deacetylation effect was more pronounced on hemicellulose digestibility in comparison to that of the cellulose digestibility showed that the cellulose content, lignin, and biomass crystallinity are the factors which regulate the impact of OAc (Pan et al. 2006).

1.3.2.4 Cell Wall Protein

The wall of the plant cell includes certain proteins that could influence the biomass enzyme hydrolysis. The cell wall proteins (Han et al. 2017) checked, which may influence cellulose hydrolysis. Such proteins can be categorized into two groups based on their positive and negative effects on biomass enzymatic hydrolysis. Cellulose hydrolysis is synergized by endogenous enzymes which include hemicellulase, cellulase, and polysaccharide wall cell debriefing enzymes like ferulic esterase and acetyl xylan esterase. Some proteins including expansion and Zea are supposed to further disrupt the wall's hydrogen binding of non-covalent proteins such as expansion and polysaccharides. Such regulators of action of the above enzymes are negative proteins in plant cell walls such as polygalacturonase inhibitors and xylanase inhibitors. During the biomass storage and pretreatment process, such type of proteins generally gets denatured and degraded. Therefore, the cell wall proteins can have minimal consequences on the enzyme hydrolysis of biomass (Han and Chen 2007).

1.4 Pretreatment Processes in LCB Valorization

The pretreatment methods reduce the recalcitrant nature of lignin and reduce the crystallinity and polymerization of the cellulose to make accessibility of the cellulose for further treatment by increasing the rate of hydrolysis. Pretreatment affects the biological, chemical, and physical nature of the biomass (Fig. 1.3). However, the ratio of lignin, hemicellulose, and cellulose varies from plant to plant, therefore the effectiveness of the pretreatment mainly depends on the type of biomass content and the type of pretreatment selected. The main aim of pretreatment methods is (1) breakage of the hydrogen, ester, and ether linkages present among the lignin, hemicellulose, and cellulose, (2) to reduce the crystallinity, (3) to enhance the surface area for accessibility of the cellulose to enzyme cellulase for hydrolysis. The pretreatment effectiveness is obtained only when there should be less use of

chemicals, less degree of denaturation of the cellulose, cost-effective, and less formation of inhibitory compounds (Baruah et al. 2018).

1.4.1 Physical Methods

The main target of physical pretreatments is to increase the substrate availability of the enzyme. This can be achieved by short processing time, less energy consumption, easy excess, and minimal formation of toxic compounds.

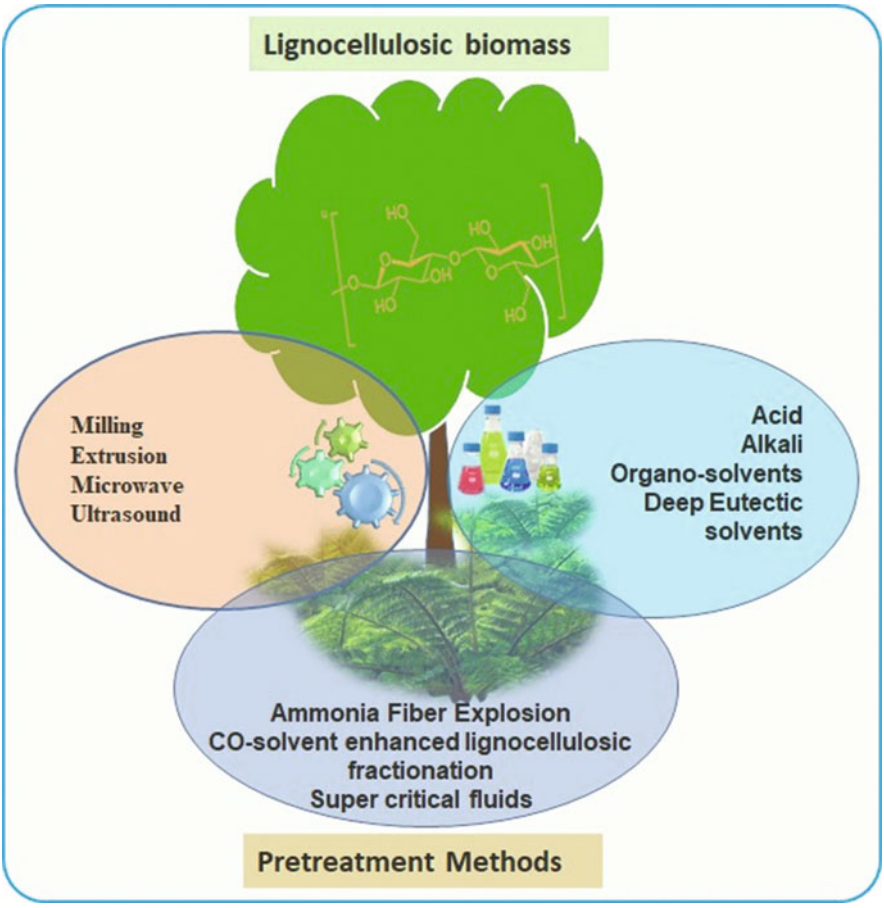

Fig. 1.3 A schematic representation of various pretreatment technologies for LCB

1.4.1.1 Milling

In the pretreatment, particle size up to 0.2 mm is considered optimal for effective milling. There are many ways of milling such as ball milling, vibro-energy milling, hammer milling, and colloidal milling. The milling decreases the particle size, degree of crystallinity, and polymerization. Ball milling and vibro-energy milling are most commonly employed to reduce particle size. Ball milling is effective for both wet and dry material and also increases the production of fermentable sugars by reducing the crystallinity index up to 74%. When oil palm empty fruit brunch treated with ball milling on an average for 120 min, the degree of crystallinity reduced from 56.1 to 9.3%, which further promotes the enzymatic hydrolysis by promoting more surface area (Zakaria et al. 2014). However, the major drawback of ball milling is high energy consumption. It takes up to 33% of energy cost throughout the whole LCB process. Ball milling mainly targets particle size and crystallinity, while unable to remove the lignin component, which makes hindrance accessibility to digestive enzymes for hydrolysis.

The combination of wet milling with other pretreatment methods accelerated the production efficiency, when palm mesocarp fiber was treated with a combination of hydrothermal following wet milling, 98% yield of glucose was obtained, and energy consumption was reduced to 9.6 MJ/kg (Zakaria et al. 2015). Similarly, treating palm oil fiber with hot compressed water and wet milling leads to 100% sugar yield. In both cases, higher temperature alters the ester linkages between lignin and cellulose, which favors more availability of surface area for further enzymatic hydrolysis (Rizal et al. 2018).

1.4.1.2 Microwave Radiation

In the method, LCB is treated in the microwave reactor closely sealed with high temperature from 160 to 250 °C, and at high-pressure steam injection, microwave irradiations are passed for the breakage of the material. The main principle of the pretreatment method is that heat generated by electric depolarization microwave promotes collision of the LCB components leading to degradation of lignin and hemicellulose providing free accessibility of cellulose for easy digestibility by the enzymes. Due to promising advantages of the method, i.e., low energy consumption, higher yield production, a short period of time, and absence of many inhibitory compounds, it is quite a popular pretreatment method. For better efficiency, it is combined with other pretreatment methods mainly alkali and ionic liquid pretreatment methods. When treated along with the alkali pretreatment, two process occurs simultaneously at a time due to heat generated by microwave, which causes expansion of the LCB components, altering structural specificity which allows easy penetration of the alkali solvents effectively removing the lignin and hemicellulose (Aguilar-Reynosa et al. 2017). When *Pennisetum polystachion* is treated with microwave-assisted alkali pretreatment NaOH, 85% of lignin is removed, which is

not obtained when treated with only alkali treatment (Tsubaki et al. 2013). When *Crotalaria juncea* fiber is treated with ionic liquid solvents assisted with microwave, 78% glucose yield is obtained due to proper removal of lignin and hemicellulose, increasing better hydrolyzing rate (Paul and Chakraborty 2018).

1.4.1.3 Pyrolysis

Pyrolysis is mainly used for the production of biofuel by decomposing the biomass into char, aerosols, and vapors. It is a widely accepted method used by the thermal industries due to less energy consumption, value-added end products, flexibility in utilization, and storage. Biomass is treated under high temperature 500–800 °C in presence of oxygen which causes thermal degradation of biomass forming biofuel and charcoal. The mild pyrolysis called torrefaction is used for increased effectiveness in the pretreatment methods. The difference between torrefaction and pyrolysis is the temperature used and the end products (Chen and Wan 2018). In torrefaction, temperatures range from 200° to 300 °C, and the end product is torrefied biomass (Santos et al. 2012).

1.4.1.4 Ultrasonification

The method is based on the principle of cavitation. The shear forces caused by cavitation which break the intermolecular bonds in LCB, releasing hemicellulose, and lignin, reducing crystallinity. In ultrasonication, the type of solvent used plays a very critical role to determine the hydrolyzing rate (Rodolfo de Melo et al. 2021). When *Eucalyptus* camaldulensis wood is treated with an aqueous soda solution, crystallinity increase from 34.7 to 35.3%, in distilled water from 32.6 to 35.5%, in acetic acid from 33.4 to 35.5% which clearly shows the critical role of the solvent (Baruah et al. 2018). The main advantage is it greatly reduces the hydrolysis time for biofuel production. When sugar beet shred was treated with ultrasonification followed by enzymatic hydrolysis, up to 780 mg/g cellulose was obtained which is 3.7 times higher than the untreated one (Ivetić et al. 2014).

1.4.2 Chemical Treatment

1.4.2.1 Acid Pretreatment

The acid pretreatment method is used for the release of fermentable sugar by avoiding two-step hydrolysis process. Here, biomasses are treated in a reactor that is resistant to corrosion and toxic acid. Acid pretreatment mainly targets the glycosidic linkage between cellulose and hemicellulose. A wide range of acids is used from inorganic acids like sulfuric acid, phosphoric acid, and hydrochloric acid to

organic acids like formic acid, maleic acid, oxalic acid, and acetic acid with distinctive effectiveness of each type.

Inorganic acid is used in two ways (1) concentrated acid (30–70%) at low temperature for a few minutes and (2) dilute acids (0.1–10%) at high temperature for a longer time period. Concentrated acid increases the hydrolysis rate 90% more but due to high concentration, it becomes toxic and corrosive and degrades the cellulose to some extent which is not favorable leading to the formation of inhibitory compounds like furfurals, phenolic acid, aldehydes, etc. Also, the degradation of cellulose indirectly affects the yield. Therefore, it needs a high maintenance level and additional biodetoxification methods to remove toxic compounds which adds up the cost.

Whereas on treating with dilute acids, the degradation rate is negligible and experimental studies indicated that dilute sulfuric acid 0.1% is the most effective acid, widely used in all pretreatment methods. When the rice straw was treated with two different methods, one with 0.4% dilute sulfuric and the other with alkali pretreatment, 10% biomass released 163 mg sugar/g in dilute acid, while only 92 mg sugar/g treated with alkali treatment (Baruah et al. 2018).

1.4.2.2 Alkali Pretreatment

The alkali pretreatment is most effective in removing the lignin in LCB as it is a simple process and energy efficient usually contains alkali like sodium hydroxide, potassium hydroxide, and calcium hydroxide. In alkalinity, the degree of crystallinity index increases due to the removal of lignin and hemicellulose, but the removal of the lignin is more effective for providing a surface area to an enzyme. The basic requirements of the alkali pretreatment method are a tank, water jacket, CO_2 scrubber, temperature controller, temperature sensor, and heating element (Rezania et al. 2020). The pretreatment method involves saponification and salvation process involving the breakage of intermolecular ester bonds among hemicellulose and lignin. Treating lignocellulose biomass with alkali solubilizes the lignin and hemicellulose in the solution making free accessibility of cellulose to hydrolyzing enzymes. Among different alkali like potassium hydroxide, sodium hydroxide, calcium carbonate, and ammonia, sodium hydroxide is the most effective alkali. For instance, when the rice straw is treated with 1% NaOH for 3 h at room temperature, it increases methane production by 34% ((Shetty et al. 2017). Another alternative solvent used is calcium hydroxide because of cost-effective and easy to handle, for example, experimentally corncob residue treated with calcium hydroxide increases the yield two times more than that treated with alkali treatment (Shah and Tabassum 2018).

1.4.2.3 Organosolv Pretreatment

Organosolv pretreatment is mainly used for the extraction of pure lignin by using an aqueous organic solvent at a temperature nearer to 200 °C in presence of the salt acid catalyst, acid, and base. For catalyst, sodium hydroxide, sulfuric acid, and magnesium sulfate are commonly used. When the biomass is treated with organic solvents in the presence of acid-base catalysis, the acid causes the hydrolysis and depolymerization of the lignin which gets dissolved in phenol and organic solvents on cooling, from which pure lignin is extracted (Borand and Karaosmanoğlu 2018). In the case of aqueous organic solvent, aqueous formasolv is used containing formic acid, water, and hydrochloric acid. Ethanosolv is also used but due to many drawbacks, it is not preferred, mainly concerned with compactivity with the hydrolysis of cellulose (Rezania et al. 2020).

1.4.2.4 Ionic Liquid Pretreatment

This type of solvent has higher thermal stability, low vapor pressure, less melting point, and high polarity. The main principle is ionic liquids compete with hydrogen bonds which causes a disturbance in the chemical bonds between lignin, hemicellulose, and cellulose. This favors the accessibility of the enzyme for hydrolysis. Ionic liquid solvents mainly target the solubility of cellulose and lignin, thus interrupting the linkage. The most striking property of the ionic liquid solvents is that they are highly reusable and renewable due to which it is widely used in recent times. A few types of ILs include imidazolium based ($[(C_3N_2)X_n]^+$), pyridinium based ($[(C_5N)X_n]^+$), pyrrolidinium based ($[C_4N)X_n]^+$, sulfonium-based $[SO_3]^+$, and others such as choline. Among these, imidazolium salts are the more frequently used ILs because solvents can be used four times with a 99% recovery rate. The yield of sugar is 3 times more with ionic liquid solvents than with alkali or any other treatments. When *Miscanthus giganteus* grass was treated with triethylammonium hydrogen sulfate, 75% of lignin and hemicellulose (100%) got solubilized in the IL solution. The results of treatment mainly depend on the characteristic of the biomass, type of anion, cation, and temperature (Zhang et al. 2017). 1-ethyl 3-methyl imidazolium is used by Li et al. (2010) for the pretreatment of switchgrass to remove lignin at 160 °C temperature for a few hours. The results show 62.9% lignin removal and increased enzyme digestibility in ionic liquid treatments. Despite being able to remove lignin content to a greater extent, it is not preferred because of the incompatibility of the cellulase with ionic liquids. In the presence of a solvent, the cellulase enzyme gets inactivated, which is not suitable for the pretreatment task (Chylenski et al. 2019).

1.4.3 Physicochemical Methods

1.4.3.1 Steam Explosion Method

The pretreatment method involves a combined action of mechanical and chemical forces simultaneously reducing the particle size and efficiently hydrolysis of the cellulose and hemicellulose for yield at a very high temperature (160–260 °C) and pressure of 0.69–4.8 Mpa at the initial stage for a few seconds to 1-min time interval. Then rapidly followed by reducing the pressure at the atmospheric level. This sudden exposure to pressure gradient causes explosive decompression by breaking the linkage between the lignin, cellulose, and hemicellulose. This allows access to the cellulase enzyme for hydrolysis by changing lignin transformation (Baruah et al. 2018). Acetic acid produced during hemicellulose hydrolysis acts as a natural catalyst that further increases the rate, therefore, called autohydrolysis. The addition of an acid catalyst increases the hydrolysis rate of hemicellulose and cellulose, thus reducing the processing time (Baruah et al. 2018). The efficiency rate of the steam explosion is inversely proportional to the moisture content, and more moisture content affects its effectiveness. The degree of solvability of hemicellulose and hydrolysis can be attained by the high temperature at a short interval or low temperature for a longer period. When the corn stover was treated with the steam explosion at 140–220 °C temperature for 2 min, it increased the yield of sugars by 22 times higher than control (Lizasoain et al. 2017). Birch samples were treated with steam at a temperature of 170–230 °C to check enzymatic hydrolysis, and outcomes showed the highest enzymatic hydrolysis (Vivekanand et al. 2013). When the 2.2% H_2SO_4 was used as a catalyst at 170 °C for 5 min ~91% of glucose yield was achieved and found to be more effective for softer woods (Meng et al. 2017).

1.4.3.2 Ammonia Fiber Expansion (AFEX)

The biomass is treated in a closed container at high temperature (60–100 °C), under very high pressure using ammonia as a catalyst. Normally 0.3–2 kg of ammonia is used, and the procedure is carried out for 5–45 min depending upon the composition of the biomass. It is rapidly followed by high pressure, causing an explosive decompression of lignin and cellulose (Lewandowski et al. 2020). Four parameters determine efficiency, i.e., moisture content, temperature, blowdown pressure, and ammonia loading. However, only 50% of lignin and hemicellulose can be removed but hydrolysis rate and yield production are very much higher with low enzymatic activity compared to other pretreatments. Zhao et al. (2014a) optimized the AFEX condition for enzyme digestibility of corn stover. The optimized condition is 5:1 ammonia, 70% of moisture content at 170 °C temperature. This comparative study had also carried out for ethanol production for corn stover between AFEX and dilute acid treatment, which showed a higher production rate at the AFEX. The cellulose

digestibility is proved to be 84% effective in removing lignin content in the pretreated *Sorghum* as compared to untreated *Sorghum,* i.e., 38% (Salvi et al. 2010).

1.4.3.3 CO_2 Explosion Method

The methods involve the treatment of LCB with supercritical CO_2 under high pressure. Due to the unique property of gas like with liquid-like solvating power at high pressure, CO_2 diffuses and changes the higher level of hemicellulose and lignin. It forms a natural catalyst, when dissolved in water, forms carbonic acid which accelerates the hydrolysis rate so most effective for moisture content. Increasing the pressure increases the hydrolysis and accessibility of cellulose (Escobar et al. 2020). Benazzi et al. (2013) treated sugarcane bagasse with supercritical CO_2, 66% fermentable sugar was obtained. Soybean hull treated with CO_2 explosion obtained 97% glucose yield (Islam et al. 2017). Another similar experiment by Yin et al. (2014) observed when corn cob and corn stalk were treated with a combination of CO_2 explosion and ultrasonic pretreatment, 75% and 13.4% of hydrolysis of the enzymatic rate increased.

1.4.3.4 Liquid Hot Water (LHW)

This method is also known as solvolysis, aqueous fraction, aqua solv, and hydro thermolysis. LHW is a modified form of the steam explosion method, which involves steam. In the LHW method, the LCB is treated with liquid water at a very intense temperature normally ranging from 170 to 230 °C with pressure up to 5 MPa. In these processes, liquid water under high temperature is brought in contact with biomass for 15 min, high pressure prevents the evaporation of the water. It is found to be very effective in sugarcane bagasse, wheat, rye straw, etc. (Baruah et al. 2018). There are three methods to carry out the process—(1) co-current pretreatment which is carried out by heating biomass slurry and water at a high temperature, holding it for a controlled residence time under pretreatment conditions, and finally applying a cool environment, (2) treatment involves the pumping of hot water against the biomass, (3) by making biomass as a stationary stage and passing hot water over it. To enhance the productivity of the yield, LHW is combined with alkali solvents using it as a catalyst. Using NaOH as a catalyst increases the productivity rate (Zhuang et al. 2016). Usage of the catalyst along with increasing yield lowers the high temperature required. Improving the production cost. When corn stover is treated at 160 °C for 10 min results in 60% of lignin removal, 53.8% hemicellulose solubilization, and 73.1% yield obtained (Mao et al. 2010).

1.4.4 Biological Pretreatment

In the living world, there is a group of organisms called fungi that can degrade lignin, hemicellulose, and cellulose without the help of any external uses. They are commonly called decomposers. They have a group of enzymes like peroxidase, laccases, chitinase, and cellulase enzyme which can degrade the biomass. Some of the genera of white-rot fungi *Ganoderma resinaceum, Pleurotus ostreatus, Pycnoporus cinnabarinus, Lepista nuda* have a higher degree of delignification and decolorization capacity (Eichlerová and Baldrian 2020). Each type of fungi is specific for degrading certain components. White-rot fungi like *Cyathus stercoreus* and *Ceriporiopsis subvermispora* are solely responsible for the delignification of *Cynodon dactylon* grass (Zhao et al. 2014b). White-rot fungus belongs to the group basidiomycetes which have the only capability to degrade the lignin naturally as the enzymes are responsible for degradation, hence termed enzymatic treatment. The regulation of enzyme activity is mainly regulated by the carbon and nitrogen source. The enzymes involved in lignin degradation are laccases, lignin peroxidase, manganese peroxidase, and versatile peroxidase. Based on the target component, the enzymes are classified as hydrolytic which targets the cellulose and hemicellulose and ligninolytic which targets the lignin part. The advantages of biological treatment are diverse no production cost, no formation of the inhibitory compounds, environmentally favorable, no investment in the operational set, no use of any expensive solvents, catalysts, the microorganism can easily be utilized from soil, air, or any agricultural waste (Shah et al. 2019).

1.5 Strategies to Enhance Enzymatic Conversion Based-Biorefinery

1.5.1 Pretreatment Regimes to Overcome LCB Recalcitrance

The majority of pretreatment regimes can effectively overcome recalcitrance by partial factors which restrict the enzymatic hydrolysis of LCB. Consequently, combined pre-remedial strategies are taken into consideration to enhance the lignin and hemicellulose digestibility. Physical, chemical, and physicochemical pretreatment show apparent effects on the elimination of hemicelluloses, while biological pre-remedy can effectively delignify from LCB (Holwerda et al. 2019). Therefore, the pretreatment techniques should be blended to enhance the digestibility of LCB and facilitate the recovery of lignin and hemicelluloses for generating valuable by-products.

Combination of the pretreatment like a steam explosion, hydrothermal with alkali treatment, biological pretreatment with physical and chemical pretreatment to reduce the degradation time by reducing the particle size and structural framework increases the efficiency and time for the biological enzymes (Yu et al. 2009). The combination

of steam explosion with ionic liquid and ultrasound pretreatment has been very effective in increasing the productivity and reducing the disadvantage factors which was a major issue in the application of the individual pretreatment methods (Selig et al. 2010). The almond tree pruning treatment with a combination of alkaline method and hydrothermal method results incomplete degradation of hemicellulose with very low content of xylan and 60% lignin solubilized with any structural change in alkaline solution which adds a plus advantage in treatment (Cuevas et al. 2014).

Alkali pretreatment is considered to be the most effective treatment due to very low cost and its high efficiency. Based on the experimental results, the combination of steam explosion with alkali pretreatment and dilute acid with hydrothermal are the most promising pretreatment combinations (Lee et al. 2015). Sun et al. (2014) treated the thermo mechanical fiber of *Eucalyptus urophylla* with the hydrothermal method in combination with NaOH alkaline solution, to monitor the enzyme digestibility efficiency. The result showed the total degradation of hemicellulose in hydrothermal, and 50–60% of lignin solubilize in the alkaline solvent with low xylan content from 8.32 to 20.85% (Cherubini 2010).

1.5.2 Challenges of Lignin Condensation and Repolymerization

The repolymerization and condensation reaction in lignin has been reported in various biomass conversions (Brittain et al. 2018). The process of lignin condensation and repolymerization involves vinyl condensation, radical coupling polymerization with reactive fragments including formaldehyde. The repolymerization with reactive useful agents consists of the free phenolic hydroxyl group (Kim and Kim 2018). The condensed form of lignin residues is less reactive as compared to their native form and, in fashionable, can most effectively be used as a source of reasonably priced energy (Renders et al. 2017). Therefore, the restoration of lignin for biomass processes in the following ways (Li et al. 2010):

1. have stability underneath acidic conditions to prevent condensation and a new era of C-C bonds;
2. include ether links (C-O) for your backbone for a whole disabled;
3. generate in a plant of a phenylpropanoid monomer simplest for simple products.

The conventional fractionation process, particularly the pretreatment of the biomass, can successfully exclude the lignin from the biomass shape, by using acid catalysts or base. The resulted product is strong lignin, especially which goes through irreversible repolymerization depending on pretreatment conditions, which restricts its ability to depend upon low-overall performance, low molecular weight products, acidic and neutral situations, the new intermolecular links (i.e., a C–C link) between the fragments of lignin with the aid of the carbohydrates, and nature of monomeric products (Kim and Kim 2018).

1.5.2.1 Undesirable Reaction Occurring During Lignin Depolymerization Process

In the lignin liquefaction technique, an enormous amount of solid waste is generated at the end of reactions, which restricts the overall performance of liquid oil. It is far believed that the synthesis of undesirable stable products of intermediate products is produced drastically in the course of the lignin depolymerizing reactions. Many response networks have been proposed to explain these secondary reactions of repolymerization and condensation. At high temperatures, the breakdown of considerable ether links (e.g., β-or 4 and α-or-or-or-4) in lignin can produce notably reactive and unstable free radicals. Those radical species, within the early level of the conversion method, can react even extra through reorganization, abstraction, and irreversible radical–radical coupling reactions to produce large molecular weight products (Kim et al. 2017).

As primary, intermediate products substituted with vinyl and allyl (for example, 4-vinyl phenol, 2-methoxy-four-vinyl phenol, isoeugenol, etc.) occur from the decomposition of thermal lignin. Due to its exceptionally reactive nature toward the polymerization and condensation reactions, to avoid undesirable reactions, the fast stabilization of the reactive intermediates is necessary, otherwise, they may be easily tapered to shape oligomers or also unwanted harmful products (Hoshino et al. 2002). Due to the cleavage at gamma carbon, the formation of formaldehyde is the problem in the repolymerization reactions occurring between lignin decomposition products and character formation (Rahimi et al. 2014). This deliberates the high reactivity of formaldehyde towards polymerization (for example, phenol-formaldehyde resin), a crosslinking reaction among phenolic and formaldehyde fragments obtained from lignin can without problems arise, ensuing in product formation of super molecular weight or char (Pineda and Lee 2016).

Furthermore, lignin-derived products of lignin fragments are formed due to secondary reactions that occur within the decomposition of lignin. Based on the structural point of view, because of the high reactivity of the AR-OH organization in lignin, its contribution to secondary reactions has sought attention. It is far believed that the AR-OH institution is tremendously reactive closer to the electrophile substitution reactions and may form reactive intermediates among Quinones depending on the reaction situations. Many of the species have been responsible for secondary repolymerization and also showed methoxy corporations during pyrolysis of lignin which affects the formation of character (Roberts et al. 2011). Various repolymerization pathways and condensation pathway shape larger molecules, which includes radical coupling, reactive fragments (e.g., formaldehyde) vinyl condensation concerning polymerization, and (four) organization useful reactive (an instance, AR-OH) brought on reactions of repolymerization. Reputedly, stable products or characters obtained during lignin depolymerization collectively affect from above response pathways (Shi et al. 2016). The practical methods of high-performance lignin depolymerization can greatly enhance the productiveness and quality of biorefinery. Even though the development of such techniques is restricted

due to the presence of inter-united carbon–carbon bonds in original lignin and also by the path through which links are formed during lignin extraction (Okuda et al. 2004).

1.5.2.2 Structural Modification of Lignin During Pretreatment to Avoid Condensation and Repolymerization

The exact structure of lignin stays doubtful, and several important structural tendencies of lignin have been diluted with upgrades in modern analytical techniques. Functional groups and links between the unit of their education and response networks involved in lignin depolymerization are included in these features. Methoxy, phenolic hydroxyl, aliphatic hydroxyl, and carbonyl corporation functional groups found in the LCB rely on biomass species and type of extraction method (Ragauskas and Yoo 2018). Out of many special practical corporations, the AR-OH group because of being the most reactive functional grouping has been fascinated by researchers (Kim et al. 2018). Primary products obtained from lignin are prone to extra addition and condensation under acidic and basic conditions forming an excessive molecular weight product (Roberts et al. 2011). Research by Kim et al. (2017) found that methyl groups (AR-OCH_3), the chemo-selective overlaying of an AR-OH group greatly decrease the secondary condensation reactions induced through the quinone of as much as 50%. In-situ detection of free radical species (e.g., radicals quinone), dilated that the radical-induced reactions are involved in lignin depolymerization. If the AR-OH is blocked by different groups, intermediate products of lignin greatly reduce the repolymerization reaction and inhibit the formation of quinone-methide. Also, in pyrolysis situations, the selective methylation of AR-OH boosts the production of aromatic hydrocarbons and blocks unpleasant secondary reactions (Kim et al. 2018). In processing of lignin depolymerization along with supercritical ethanol, ethanol which stabilizes the primary products by acting as a hydrogen donor solvent is no longer a functional hydrogen donor and also forms O-alkylation of AR-OH group which inhibits the process of repolymerization (Huang et al. 2017).

1.5.2.3 Recent Advancements in Capping Agents

In recent times, scientists have successfully integrated the usage of the capping agents to stabilize the reactive sites or any intermediate formed during lignin fragmentation in LCB. One of the most commonly used capping agents is boric acid which contains AR-OH groups to catalyze lignin and form boric ester during depolymerization (Toledano et al. 2014). The boric acid suppresses the unwanted condensation reactions and increases oil production (Roberts et al. 2011).

Along with boric acid, phenol has also been tested thoroughly to be used as a tapping agent to stabilize the formaldehyde and phenolic moiety. Additionally, phenol has also been capable enough of reducing the lignin of characters and

residues forming a phenolic compound under the presence of lignin catalyzed lignin depolymerization process. The hydrolytic degradation of lignin below the combination of water-ethanol under the usage of phenol as a plugging agent reduced the individual yield up to 70% (Liu et al. 2015). Some reaction conditions are carefully accomplished for the optimal LCB conversion like the long response time, high temperature, and phenol ratio to the lignin content to avoid repolymerization. The volatile radical species and unstable intermediates are formed during the depolymerization that might leave low molecular weight phenols as byproducts (Trajano et al. 2013; Luterbacher et al. 2015).

1.6 Conclusion

LCB has the potential to be used as an alternate fuel and other biochemicals to sustain a promising future human need. However, the LCB valorization compromises due to structural and functional recalcitrance, mainly the lignin, in the complex heterogeneous biomass. From the above discussion, it is clear that physicochemical factors and phenolic hydrolysis of lignin during enzymatic reactions are primarily responsible for the recalcitrance. The application of hybrid pretreatment techniques and strategic work on the lignin biosynthesis pathway understands cell wall recalcitrance and ways to reduce it.

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

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Chapter 2
Insight into Various Conventional Physical and Chemical Methods for the Pretreatment of Lignocellulosic Biomass

Bharat Manna, Manali Das, Pradipta Patra, and Amit Ghosh

Abstract The generation of renewable energy resources as an alternative to fossil fuels is essential to sustain the growing human population. Lignocellulosic biomass is considered an important renewable resource for various value-added compounds and biofuels, as the world is currently poised toward a carbohydrate-based economy. Analogous to petroleum refineries, biorefineries deal with the carbohydrate polymers (cellulose, hemicellulose) and aromatic compounds (lignin), which can be processed into different bioproducts. However, the complex architecture of crystalline cellulose, hemicellulose, and lignin creates high recalcitrance, which requires significant pretreatment steps. Thus, developing cost-effective pretreatment is crucial for the effective separation of the biomass components. In this chapter, first, the basic components of the lignocellulosic biomass have been briefly described followed by the various conventional physical and chemical pretreatment methods. In addition, the efficiency of different biomass-specific pretreatment operations and their combinations has been discussed in detail. Moreover, challenges of the pretreatment processes, like chemical recovery, inhibitory byproducts formation, prolonged and costly methods, and feedstock utilization are also highlighted. Overcoming the challenges has demonstrated the potentiality of the available pretreatment methods in the advanced biological refinery process for the production of biofuels and various value-added compounds.

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Abbreviations

AFEX	Ammonia Fiber Explosion
CrI	Crystallinity index
DA	Dilute acid
DP	Degree of polymerization
GR	Gamma irradiation
ILs	Ionic liquids
LCB	Lignocellulosic biomass
LHW	Liquid hot water
MWR	Microwave radiation
OS	Organosolvent
SE	Steam explosion
WO	Wet oxidation

2.1 Introduction

Environmental pollution and climate changes are two major challenges of the present century, necessitating the use of non-fossil-based carbon-neutral fuel resources. Lignocellulose biomass (LCB) based feedstock presents one of the most suitable alternative resources for biofuels and value-added bioproducts in a sustainable manner. LCB has gained huge attention for promising biorefinery feedstock because of its ample abundance and lower cost than other biomass resources. However, conversion of LCB into useful biofuel or other value-added chemicals takes the integration of a series of chemical as well as biological procedures (Fig. 2.1). Considerable obstacles are involved in the effective utilization of LCB. The major hindrance to the conversion process is the complex organization of the structural components in the lignocellulosic biomass. The primary components of LCB are cellulose $(C_6H_{10}O_5)_n$, hemicellulose $(C_5H_8O_4)_m$, and lignin $[C_9H_{10}O_3(OCH_3)_{0.9–1.7}]_x$ (Akhtar et al. 2016; Jørgensen et al. 2007; Kumar and Verma 2020a). This integral complexity of the plant cell wall leads to major recalcitrance against any kind of deconstruction of the LCB.

Pretreatment is the essential method performed upstream of the LCB to biofuel conversion, in order to overcome the recalcitrance and disrupt the complex organization of cellulose, hemicellulose, and lignin. Pretreatment is a vital component in converting LCB into liquid fuels and chemicals. Pretreatment aims to separate aromatic lignin and polysaccharides (cellulose and hemicellulose) and enhance accessibility to the hydrolytic enzymes for saccharification. The more the LCB is

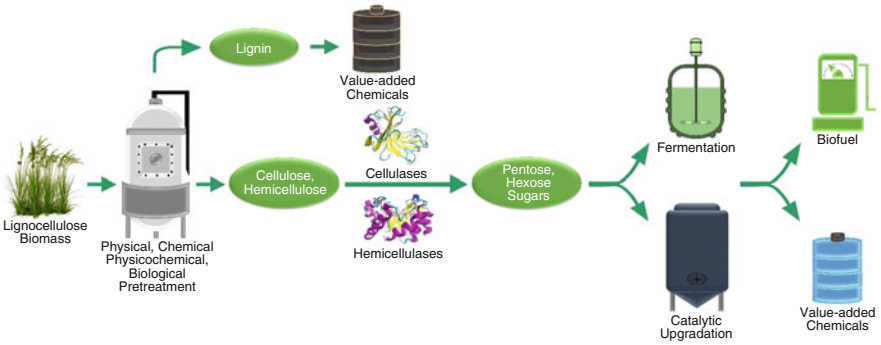

Fig. 2.1 Schematic representation of the general pathway for biofuel and value-added chemical production in lignocellulose biorefinery. The lignocellulosic biomass undergoes steps like pretreatment and enzymatic hydrolysis for removal of lignin and depolymerization of cellulose and hemicellulose, releasing simple sugars for catalytic processing or fermentation for the production of biofuel or value-added chemicals

susceptible to hydrolysis, the more yield will be achieved for the fermentation process. Different types of pretreatment processes are employed to overcome the recalcitrance and breakdown of crystalline cellulose, opening the hemicellulose-cellulose surface with improved accessibility for further chemical conversion (Bhutto et al. 2017; Chen et al. 2017; Agrawal and Verma 2020; Bhardwaj et al. 2021). Notably, each pretreatment method has its specific consequence on the structure of the cellulose, hemicellulose, and lignin of the LCB. The physical and chemical changes of the LCB fractions are directly linked to the overall operation and effectiveness of the downstream process in terms of hydrolysis, substrate solubilization, fermentation rate, mixing, ethanol yield, etc. Thus, the choice of proper pretreatment method based on the LCB feedstock type and its effects on downstream steps is essential for the overall production of biofuels and chemicals.

In this chapter, first, the components of the LCB have been summarized followed by a discussion of the various conventional physical and chemical pretreatment methods. The challenges of different pretreatment methods are also highlighted along with the production of biofuels and various value-added compounds. Understanding the overall process helps in selecting the proper pretreatment method with subsequent operations based upon the type of feedstock, hydrolysis, and fermentation steps.

2.2 Components of Lignocellulosic Biomass

Plant cell wall mainly comprises polysaccharides such as cellulose, hemicellulose, aromatic polymer lignin, and a trace amount of pectin (Fig. 2.2) (Ververis et al. 2004). These complex composite materials provide the basic structural support to the plants. Cellulose remains in the core, surrounded by hemicellulose, whereas lignin

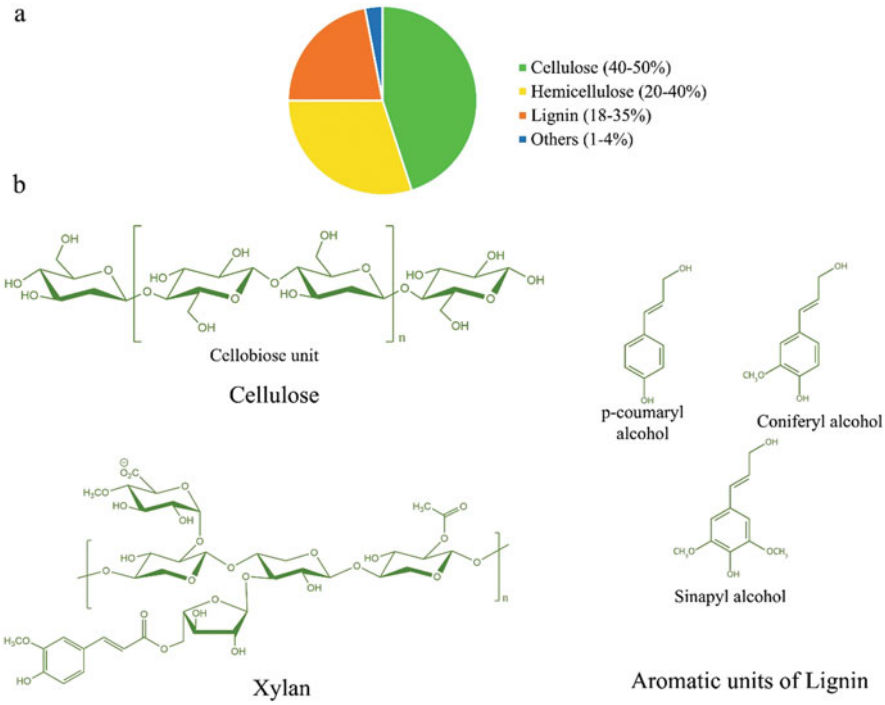

Fig. 2.2 Composition of the lignocellulosic biomass: (**a**) Cellulose (40–50%), hemicellulose (20–40%), and lignin (18–35%) along with a trace amount of pectin, protein, extractives, and ash make up the lignocellulosic biomass. (**b**) Chemical structures of the major constituents of lignocellulosic biomass. Cellulose is a homopolymer of β-1,4-glycosidic linked glucan chains, whereas xylan is hemicellulose composed of β-1,4-linked xylose residues. Lignin is an aromatic compound consisting of mainly p-coumaryl, coniferyl, and sinapyl units

appears at the outermost part of the plant material. Cellulose is a linear homopolymer of β-1, 4 linked glucose, whereas hemicellulose is a branched heterogeneous polysaccharide consisting of monomers like xylan, arabinoxylan, and others. Pectin is made up of complex polysaccharides such as α-linked galacturonic acid and rhamnose monomers. Lignin is also heterogeneous in nature and mainly contains alkyl-aromatic units forming a branched polymer. The composition of some popular lignocellulosic plant biomass is given in Table 2.1.

2.2.1 Cellulose

Cellulose is the most abundant organic matter available on earth, which represents the primary constituent of the plant cell wall. Out of all the components of the lignocellulosic biomass, cellulose represents a vast amount of fermentable sugar

Table 2.1 Composition of some industrially important lignocellulosic biomass including agricultural wastes which can serve as potential lignocellulosic biomass sources in biorefineries

Plant material	Major components of lignocellulosic biomass			References
	Cellulose (wt %)	Hemicellulose (wt %)	Lignin (wt %)	
Alfa Alfa	21.8	12.4	9.7	Dijkerman et al. (1997)
Bermuda grass	47.8	13.3	19.4	Li et al. (2009)
Corn cobs	35–39	38–42	4.5–6.6	Okeke and Obi (1994)
Cotton seed hairs	80–95	5–20	0	Howard et al. (2003)
Hard Woods	45 ± 2	30 ± 5	20 ± 4	Kuhad et al. (1997)
Maize	35	16.8	7	Kasthuraiah and Sai Kishore (2017)
Miscanthus	45	27	12	Kasthuraiah and Sai Kishore (2017)
Reed canary straw	42.6	29.7	7.6	Bridgeman et al. (2008)
Rice straw	41	21.5	9.9	Lee (1997)
Soft woods	42 ± 2	27 ± 2	28 ± 3	Kuhad et al. (1997)
Sugar cane	40	27	10	Kuhad et al. (1997)
Sweet sorghum bagasse	34–45	18–28	14–22	Saini et al. (2015)
Switchgrass	45	31	12	Howard et al. (2003)
Wheat straw	44	29.6	10.4	Bridgeman et al. (2008)

feedstock and a major focus of biofuel and value-added chemical generation on an industrial scale. However, it is more recalcitrant to the catalytic deconstruction process than other carbohydrate polymers. Cellulose is a linear chain of homopolysaccharides with anhydrous glucose units connected by β-1,4-glycosidic bonds.

Dimerization of glucose monomers through β-1,4-glycosidic bond forms cellobiose which is the basic structural unit of cellulose. The spatial organization of the β-1,4-glycosidic bonds inside the glucan chains gives rise to specific inter and intramolecular hydrogen bonding interactions imparting high crystallinity, making it insoluble in water or other common solvents (Bishop 2015; Kumar and Verma 2020b). Also, each cellulose chain comprises a reducing end at one end and a non-reducing end at the other (Fig. 2.3). During chain elongation, new glucose monomers are added to the non-reducing end. Proper knowledge of cellulose crystallinity is crucial for understanding the depolymerization process. The β-1,4 linked glucan chains form a highly ordered crystalline structure. It is reported from the X-ray diffraction data that the crystalline microfibrils contain 24–36 linear chains (Endler and Persson 2011; Fernandes et al. 2011). In some cases, loss of crystallinity results in disordered regions, which are known as amorphous cellulose. The highly ordered crystalline cellulose is separated by such amorphous regions. Interchain as well as intrachain hydrogen bondings due to the spatial organization of the hydroxyl groups of the glucose along with the different pyranose ring arrangements in the

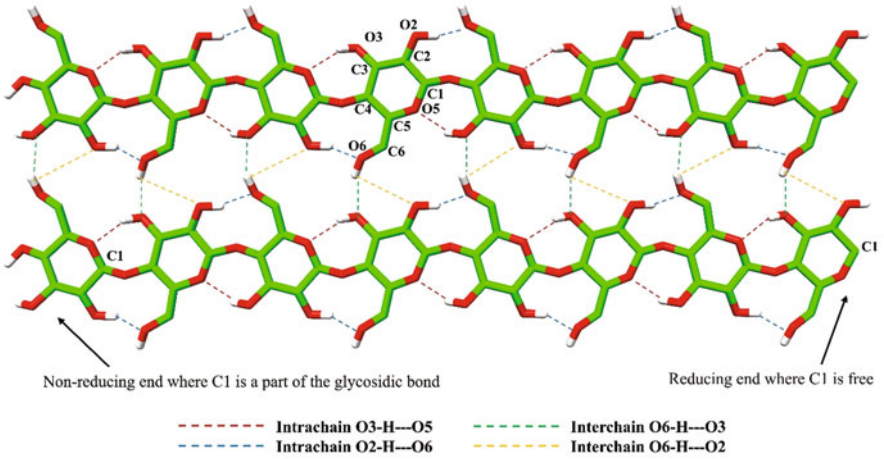

Fig. 2.3 Inter and Intra-hydrogen bonding in cellulose. Intrachain hydrogen bonding forms between O2H2···O6 and O3H3···O5, whereas interchain interaction occurs via O6H6···O3 hydrogen bonding between two cellulose chains. Carbon, oxygen, and hydrogen are shown in green, red, and white, respectively

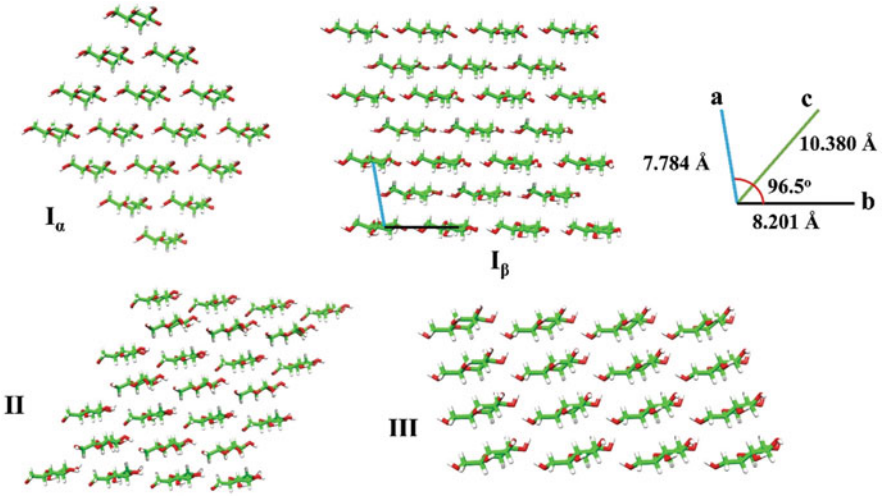

Fig. 2.4 Crystal structures of cellulose allomorphs. The end-on view of different cellulose crystals is shown. Carbon, oxygen, and hydrogen are shown in green, red, and white, respectively. The unit cell parameters for cellulose I_β are also presented

cellulose crystal give rise to four distinct crystalline allomorphs of cellulose. These are designated as cellulose I, II, III, and IV, respectively (Fig. 2.4). The difference is based on the crystal unit cell parameters such as unit cell length and unit cell angles, which defines the packing of cellulose chains in the crystal (Table 2.2).

Table 2.2 Different types of crystalline cellulose and their unit cell parameters

Cellulose crystal	Unit cell type	Unit cell parameters	References
I_α	Triclinic	a = 6.717, b = 5.962, c = 10.400, α = 118.08°, β = 114.80°, γ = 80.37°	Nishiyama et al. (2003)
I_β	Monoclinic	a = 7.784, b = 8.201, c = 10.380, γ = 96.5°	Nishiyama et al. (2002)
II	Monoclinic	a = 8.10, b = 9.04, c = 10.36, γ = 117.1°	Langan et al. (2001)
III	Monoclinic	a = 10.25, b = 7.78, c = 10.34, γ = 122.4°	Sarko et al. (1976)
IV	Orthogonal	a = 10.25, b = 7.78, c = 10.34	Gardiner and Sarko (1985)

Among all the cellulose allomorphs, cellulose I is predominantly found in nature. It is basically a mixture of two cellulosic forms, i.e., I_α and I_β (Fig. 2.4). The I_β form of cellulose has a monoclinic unit cell and is mostly found in higher plants like wood, cotton, and animals, whereas the I_α form has a triclinic unit cell and is found in a wide range of microorganisms like bacteria (*Acetobacter xylinum*) and algae (*Glaucocystis nostochinearum*). The cellulose I_β form is gaining more interest, as this form is abundant in higher plants and serves as a potential feedstock for fermentable sugar generation. Cellulose II can be obtained from cellulose I by either alkali treatment or solubilization and regeneration (Mittal et al. 2011). The unit cell in cellulose II is also monoclinic as the I_β. However, the chains in cellulose I_β are parallel, whereas the chains in cellulose II are antiparallel. Cellulose I or II can be converted to cellulose III form by ammonia pretreatment which is more amorphous in nature. Allomorph III is subcategorized into cellulose III_I or III_{II} based on the source of the conversion process. Due to reduced crystallinity, both the II and III allomorphs have greater enzyme accessible surface area that enhances the saccharification process (Chundawat et al. 2011; Payne et al. 2015). Cellulose IV can be produced by high-temperature treatment of cellulose III. This form of cellulose is less characterized. However, reports suggest that it is structurally similar to cellulose I_β (Nishiyama 2009).

Some crucial properties of cellulose that influence its pretreatment and enzymatic scarification are described below:

(a) *Crystallinity index (CrI):* It provides a measure of the compactness and reactivity of the cellulosic material which can be quantified by an X-ray diffraction pattern. For example, the *CrI* for cellulose type I can be obtained using the following method of Jayme and Knolle (Lenz 1994):

$$CrI = 1 - \frac{h_{am}}{h_{cr}} = 1 - \frac{h_{am}}{(h_{tot} - h_{am})} \tag{2.1}$$

where h_{cr} is the height of the cellulose crystal in the 002 reflections with $2\theta = 22.5°$, h_{am} is the height of the amorphous cellulose, and h_{tot} is defined as:

$$h_{tot} = h_{cr} + h_{am} \tag{2.2}$$

(b) *Degree of Polymerization (DP):* The degree of polymerization is another important parameter affecting enzymatic hydrolysis. It determines the availability of terminal and internal glycosidic bonds for both the exo- and endo-catalytic enzymes. Three specific DPs are generally considered such as the number average DP (DP_N), the weighted average DP (DP_W) and DP obtained from viscosity (DP_V). The governing formulae are given below:

$$DP_N = \frac{M_n}{MW_{glu}} = \frac{\sum N_i M_i}{\sum N_i} / MW_{glu} \tag{2.3}$$

$$DP_W = \frac{M_w}{MW_{glu}} = \frac{\sum N_i {M_i}^2}{\sum N_i} / MW_{glu} \tag{2.4}$$

$$DP_V = \frac{M_v}{MW_{glu}} = \frac{\sum N_i \eta}{\sum N_i} / MW_{glu} \tag{2.5}$$

where M_i is the molar mass of a given fraction I with N_i number of moles with viscosity η and M_N, M_W, M_V, MW_{glu} being the average molecular weight, weight-average molecular weight, viscosity-average molecular weight, and molecular weight of anhydroglucose, respectively. Cellodextrins with DP ranging from 2 to 6 are generally water soluble (Klemm et al. 1998). Solubility decreases with increasing DP of the polysaccharide. For example, glucan chains with DP 7–13 are weakly soluble (Zhang and Lynd 2003). However, DP greater than 30 is insoluble in common solvents.

(c) *Accessibility*: Cellulases need to bind effectively to the solid surface with proper orientation of the cellulose for the initiation of hydrolysis. The microcrystalline structure of cellulose along with particle size and shape determines the probability of interaction between the enzyme and the glycosidic bonds. Thus, cellulose accessibility is a key factor in the saccharification step.

2.2.2 Hemicellulose

It is a complex branched biopolymer of pentose (C5) and hexose (C6) sugars, mostly D-xylose, D-galactose, L-arabinose, D-mannose, and D-glucose, having a short length of around 50 to 200 units. However, the composition of hemicellulose varies from different sources due to its heterogeneous nature. Hemicellulose isolated from the cell wall of rice endosperm reveals that it is composed of mainly xylose, arabinose, and glucose forming the polysaccharide moieties xyloglucan, β-glucan, and arabinoxylan, respectively (Shibuva and Misaki 1978; Bhardwaj et al. 2020; Bhardwaj and Verma 2021). The β-glucan contains both the 1,3 and 1,4 glycosidic linkages. The xylose is attached to the C6 position of the 1,4 linked glucan backbone to form the xyloglucan. The arabinoxylan shows a complex branched structure

where the arabinose is connected to the 1,4 linked xylose chain. Acetate groups are attached to the hydroxyl functional groups of the sugar rings via an ester linkage. Hemicellulose mostly provides the bridge between core cellulosic parts with outer lignin. Researchers are focusing on hemicellulose for the production of value-added chemicals in biorefineries (Rambo et al. 2015).

2.2.3 Lignin

Lignin is a non-carbohydrate phenolic polymer, made up of a complex network of aromatic alcohols. The main components of this crosslinked heteropolymer are p-coumaryl, coniferyl, and sinapyl alcohols (Rubin 2008). The units are known as p-hydroxyphenyl, guaiacyl, and syringyl when present in a polymer chain. Lignin is bound to hemicellulose with covalent linkages to form an amorphous matrix that surrounds core cellulose microfibrils. Strong ether (C–O–C) and carbon–carbon (C–C) bonds in the lignin provide high mechanical strength to the plants. So far, lignin is unusable in the fermentation process for biofuel production and can be used as an alternative resource for value-added chemical production in the biorefinery (Radhakrishnan et al. 2021; Kumar et al. 2020).

2.3 Different Technologies for Lignocellulosic Biomass Pretreatment

For the production of lignocellulosic biofuels and other economically important chemicals, the first step is to overcome the heterogeneity of the raw biomass in a cost-effective manner. Several pretreatment techniques have been adopted to separate the cellulose from other biopolymeric mixtures followed by decrystallization of the cellulose microfibrils that will be subjected to the enzymatic hydrolysis in the subsequent steps. The primary goals of the pretreatment are the efficient separation of lignin, hemicellulose, and cellulose by disrupting the structural linkages and increasing the accessibility to the hydrolytic enzymes. Moreover, some other key targets in the pretreatment process are a reduction in the crystallinity index, the degree of polymerization of the cellulose, and particle size of biomass. Some of the key criteria for a "good" pretreatment method are (a) less degradation of the biomass, (b) lowering the inhibitory product formation, (c) minimizing energy input, (d) low-cost pretreatment agents and catalyst recycling, (e) overall cost-effectiveness. The pretreatment procedures can be broadly classified into three major categories: physical, chemical, and biological pretreatments. The chemical pretreatments are subcategorized into biochemical and thermochemical processes. The traditional physical and chemical pretreatment methods are discussed in the following sections.

2.3.1 Physical Pretreatment Process

The physical pretreatment processes are aimed to increase the overall surface area of the biomass and reduction in the particle size which in turn decreases the degree of polymerization of the material. The commonly available physical pretreatment methods are described below.

2.3.1.1 Milling

Milling is a mechanical process for lignocellulose pretreatment. The different types of milling used for the deconstruction of lignocellulosic biomass are ball milling, disk milling, two-roll milling, colloid milling, and hammer milling (Mood et al. 2013). The average particle size obtained from the milling process is around 10–30 mm. However, the high energy requirement of the process raises the production cost which is partially overcome by wet disk milling with comparatively lower energy consumption. In a comparative study on the effectiveness of wet disk and ball milling processes, it was found that the total glucose and xylose yield in the enzymatic hydrolysis step from the wet disk pretreated sugarcane bagasse were 49.3% and 36.7%, respectively, whereas the ball milling pretreated biomass gave rise to maximum yield of 78.7% and 72.1%, respectively (Hideno et al. 2009). Value-added chemicals such as methyl levulinate were made with a maximum yield of 64.92 mol%, using ball milling pretreatment by facilitating cellulose depolymerization (Chen et al. 2019).

2.3.1.2 Extrusion

Extrusion is another thermo-physical pretreatment method, where the biomass is passed through an extruder with moderate to high temperatures. The rapid mixing and excessive shearing in the chamber lead to the breakdown of the crystalline structure and produce short fibers which increase the enzymatic accessibility of the biomass in the hydrolysis step. For example, extrusion with temperature up to 80 °C, screw speed of 350 rpm, and moisture content of 40% provided a yield of 94.8% glucose conversion by the enzymatic hydrolysis from the pretreated soybean hulls (Yoo et al. 2011). Recently, extrusion has been combined with cellulases for obtaining sugar conversion yield of at least 94% at 50 °C (Gatt et al. 2019). This process doesn't produce any effluent which is an advantage in terms of disposal cost reduction. Continuous operating process without washing and the possibility of easy scale-up have made extrusion an industrially important pretreatment process.

2.3.1.3 Pyrolysis

Pyrolysis is the process of deconstruction of lignocellulosic biomass at high temperatures. This pretreatment approach has been adopted by many industries. When lignocellulosic materials are subjected to temperatures as high as 300–800 °C or more, the cellulose crystallinity is gradually decreased with the production of residual char and gaseous compounds (Fisher et al. 2002). For example, 50–55 (wt %) liquid fraction, 25–27 (wt%) char, and other gaseous compounds were produced from hardwood pyrolysis at a temperature of 200–275 °C for 30–45 min followed by 450 °C for 1 h (Ortega et al. 2011). However, the decomposition is significantly less at lower temperatures. It is reported that more than 80–85% cellulose conversion can be achieved by coupling mild acid hydrolysis with pyrolysis. Cellulose decomposition can also be performed by introducing sodium carbonate or zinc chloride as a catalyst in the process. Catalytic pyrolysis shows a significant rise in the organic liquid yield of 42 wt% in pretreated biomass than raw biomass at a process temperature of 600 °C (Persson and Yang 2019).

2.3.1.4 Microwave

The complex organization of cellulose and other components of the lignocellulosic biomass can also be subjected to microwave irradiation rather than conventional heating for the deconstruction process. Microwaves produce high-frequency electromagnetic field that generates heat when interacting with an object containing mobile charges. The difference with conventional heating is that heat transfer occurs through the surface of the objects, whereas microwave introduces high molecular collision in the cellulose because of dielectric polarization which leads to the breakdown of the cellulose ultra-structure, separation of lignin/hemicellulose and finally enhancing the accessibility of the material for the hydrolytic enzymes. For example, 30% and 12% lignin removal were obtained in corn straw and rice husk, respectively, with microwave pretreatment (Diaz et al. 2015). Currently, microwave-assisted pretreatment (with irradiation at 800 W and 152 °C for 45 s) was carried out using choline chloride and lactic acid deep eutectic solvent (ChCl: LA DES) where 85%–87% pure lignin was recovered while the cellulose existed in the solid fraction (Chen and Wan 2018). In another study, researchers have reported microwave pretreatment using choline chloride and oxalic acid dihydrate deep eutectic solvent (microwave irradiation at 800 W and 80 °C for 3 min) with a pure lignin recovery of 96% (Liu et al. 2017a).

2.3.1.5 Freeze Pretreatment

Freezing treatment is a newly introduced approach where the material is stored in the refrigerator for a specific time which significantly increases the cellulase

performance before the hydrolysis step. It is found that the enzymatic hydrolysis was enhanced from 48% to 84% on a freeze pretreated rice straw (Chang et al. 2011). Low environmental impact and absence of chemical hazards make the process promising.

The main advantage of the physical pretreatment method is that the particle size of the biomass is reduced which enables efficient heat and mass transfer during further processing. On the other hand, cellulose crystallinity and degree of polymerization are decreased, thus providing higher surface area and pore size during the hydrolysis step for improved sugar yield. However, the major limitations of these processes are the high energy requirement and equipment maintenance cost which make them economically inefficient.

2.3.2 Physico-Chemical Pretreatment Process

2.3.2.1 Steam Explosion

Steam explosion is a thermo-mechano-chemical pretreatment method that involves the thermal steam-heating to degrade the lignocellulosic components, the mechanical shearing force due to sudden pressure drop, and chemical autohydrolysis for glycosidic bond cleavage. In this method, the biomass is subjected to saturated steam at a temperature of around 160 °C–260 °C at a high pressure of ~0.69 to 4.83 MPa for a few seconds to minutes followed by exposing it to the atmospheric pressure. This explosive decompression results in the disintegration of the lignocellulosic matrix. Hemicellulose hydrolysis along with lignin transformation at high-temperature treatment exposes the cellulose surface to the cellulolytic enzyme machinery in the subsequent steps. The efficiency of this process depends on the particle size, temperature, the residence time of the material, and total moisture content inside the chamber. The efficiency of the process can be greatly enhanced by the introduction of catalysts such as sulfuric acid (H_2SO_4), sulfur dioxide (SO_2), or carbon dioxide (CO_2) which accelerate hydrolysis and removal of hemicellulose. Steam explosion pretreatment at 177 °C for 5 min of banana lignocellulosic biomass gave rise to 91% glucose yield in enzymatic hydrolysis (Guerrero et al. 2017). Moreover, pretreatment including wheat straw, miscanthus, and poplar has shown the role of steam explosion in reducing hemicellulose content and breaking down the lignin cross-linkages for improved enzymatic hydrolysis (Auxenfans et al. 2017). The main advantage of this technology is that due to the partial lignin and hemicellulose solubilization, the sugar yield is improved during the enzymatic hydrolysis. On the other hand, no requirement of recycling and less environmental impact make this process industrially feasible. However, there is a possibility of forming fermentation inhibitors at high temperatures. In case of incomplete deconstruction of the biomass, soluble lignin may get precipitated and hinder effective hydrolysis. Considerable energy requirement due to the maintenance of high temperature and pressure increases the overall cost of the process.

2.3.2.2 Ammonia Fiber Explosion (AFEX)

It is a thermochemical pretreatment method that is based on the same principle as steam explosion. Here, the lignocellulosic biomass is treated with liquid ammonia at high pressure (1.72–2.06 MPa) and moderate temperature (60 °C–120 °C) for a specified time (~30 min). Then the pressure is swiftly dropped which results in the quick expansion of the compressed ammonia gas that breaks the matrix of the lignin–carbohydrate complex followed by degradation of the fibers. Ammonia pretreatment produces solid materials, whereas steam explosion gives rise to the slurry. It is notable that AFEX doesn't release any sugar due to less solubility of hemicellulose but it disintegrates the components to increase the surface area of the biopolymeric mixture for higher enzymatic accessibility. AFEX pretreatment is proven to be more effective for the pretreatment of low lignin-containing biomass such as alfalfa stems, wheat straw, rice straw, switchgrass, corn stover, etc. than hardwood and softwood materials with more lignin. AFEX process optimization can be done by varying the following parameters, i.e., the amount of ammonia to biomass loading ratio, water loading, temperature, the residence time of the biomass, and the moisture content. For example, ammonia to biomass loading ratio of 2:1 along with 120% moisture content and 5 min of residence time at 140 °C temperature were found to be optimal for bioethanol production from sorghum bagasse (Li et al. 2010). In a recent study, the efficacy of both the steam explosion and AFEX was explored for the production of animal feeds and biofuel feedstocks in a sugarcane-based biorefinery approach. It was shown that up to 3368 L and 4360 L of ethanol per hectare of sugarcane cultivated land could be generated from steam explosion and AFEX pretreated sugarcane residues, respectively (Mokomele et al. 2018). AFEX increases the effective surface for enzymatic hydrolysis and enhances the sugar yield. Besides, no inhibitory by-products are formed during the process, and no biomass washing or detoxification steps are required. However, this pretreatment method is not effective for high lignin-containing biomass and softwoods. The corrosive and hazardous properties of AFEX pretreatment require a controlled environment and costly equipment. The cost of ammonia is another concern that makes the process highly expensive at large scale (Agbor et al. 2011).

2.3.2.3 CO_2 Explosion

The CO_2 explosion is another explosion-based method similar to steam explosion and AFEX for the pretreatment of lignocellulosic biomass. In this process, supercritical CO_2 is used with a temperature and pressure greater than its critical temperature and pressure values (31 °C and 7.4 MPa, respectively). At this condition, liquid and gases can coexist, and CO_2 shows liquid-like density as well as gas-like transport properties with the ability to penetrate the lignocellulosic pores facilitating the deconstruction process. When it is used along with water, carbonic acid is formed which accelerates the polymer hydrolysis. After mild treatment, explosive release of

CO_2 due to the pressure drop destroys the architecture of hemicellulose and lignin and exposes enzyme accessible surface area for cellulose hydrolysis. It is shown that significantly higher amounts of reducing sugar (84.7%) are obtained from supercritical CO_2 (165 °C and 21.37 MPa for a residence time of 30 mins) pretreated aspen wood than the untreated biomass (14.5%) in the hydrolysis process (Kim and Hong 2001). In a recent study, supercritical CO_2 (50 °C–80 °C and 17.5–25.0 MPa pressure for 12 h–60 h) has been used for pretreating corn stover and corn cob, which resulted in around three to four-fold higher sugar yield than the untreated feedstocks during the enzymatic hydrolysis (Zhao et al. 2019). Low operating temperature with no inhibitory/toxic product formation and complete removal of CO_2 by depressurization have made this process promising for scale-up purpose. The CO_2 can be readily obtained as a by-product from many industrial processes. However, the low process efficiency for different lignocellulose resources and high-pressure equipment rises the capital cost significantly (Agbor et al. 2011).

2.3.2.4 Liquid Hot Water Pretreatment

Liquid hot water (LHW) pretreatment is an efficient pretreatment method for improving enzymatic hydrolysis in a chemical-free manner for biomass conversion. In this process, liquid water is used as a reaction medium at a high temperature (160 °C–220 °C), and pressure is controlled to keep the water in a liquid state. This method has been shown to be effective for the pretreatment of sugarcane bagasse, corncobs, corn stover, wheat straw, etc. For example, wheat pretreatment using LHW gave rise to glucose and xylose yields of 91% and 80%, respectively (Pérez et al. 2008). In another study, alkali-catalyzed (0.5% aqueous NaOH at 170 °C) LHW pretreated bamboo yielded 30.9 g/100 g reducing sugars during hydrolysis and 9.6 g ethanol/100 g bamboo after fermentation (Yang et al. 2019b). LHW pretreatment with corncobs for 10 min at 160 °C significantly enhanced solubilization of hemicellulose (pentose yield of 58.8%) and lignin removal (60%), resulting in 73.1% glucose recovery in the hydrolysis step (Imman et al. 2018). This is an environment-friendly approach with less or no inhibitory by-product formation due to low process temperature and results in significant sugar recovery from both hemicellulose and cellulose. But, there is high energy and water requirement, which limits the application of the process and it needs further optimization for commercial applications.

2.3.2.5 Irradiation

Irradiation is another approach for lignocellulosic biomass pretreatment. This is generally coupled with other conventional pretreatment processes. Application of electron beam or gamma (γ) rays along with mechanical crushing, thermal or chemical pretreatments has shown enhanced enzymatic hydrolysis in comparison with pretreatment in absence of such irradiation. Pretreatment with an electron beam

decreases the cellulose crystallinity and increases the enzymatic hydrolysis. In the case of ionizing irradiation, long- and short-lived radicals are formed that accelerate the deconstruction of biomass by chain scission and cross-linking (Khan et al. 2006). Hydrothermal pretreatment of sugarcane bagasse along with a 50 kGy electron beam has shown to increase the saccharification yield of glucose by 20% (Duarte et al. 2012). Application of electron beam (7.5 kGy) in mild alkali soaked *Artemisia ordosica* gave an increased yield of reducing sugars as 520.67 mg/g under optimal conditions (Xiang et al. 2017). In a recent study, significantly reduced particle size and low shear rate were achieved using γ-irradiation at 800 kGy along with thermal or dilute acid pretreatment where the sugar yield was found to be around 251 g/L (Liu et al. 2017b). Though electron beam or gamma (γ) rays enhance the enzymatic saccharification, the industrial application of irradiation is limited due to the high cost of installation and environmental concerns. However, these technologies can be used in combination with other methods for designing an optimized pretreatment process.

2.3.2.6 Ultrasonication

Ultrasonic radiation can also be used to deconstruct the complex architecture of lignocellulosic biomass. The basic mechanism of high-frequency ultrasonication in any liquid is the formation of bubbles followed by its sudden collapse at the solid surface, inducing a microjet streaming known as asymmetric cavitation. These cavities enhance the enzymatic accessibility at the substrate surface. At a fixed level of enzyme and substrate concentration, sonication at 40%, 60%, and 80% amplitudes (I_{diss}: 16.2, 32.2, and 43.4 W/cm^2, respectively) has shown increased enzymatic hydrolysis by 15%, 24%, and 54%, respectively, in comparison to the mechanical agitation alone (Szabo and Csiszar 2017). Ultrasonication can also be used along with other chemical pretreatment agents such as acid-alkali, ionic liquids, etc. For example, ultrasonication in combination with HCl pretreatment resulted in significantly higher delignification in deenanath grass (80.4%) and hybrid napier grass (82.1%) than that of acid pretreatment alone (Mohapatra et al. 2017). Another study shows that ultrasonication pretreatment along with NaOH in rice straw can degrade hemicellulose and lignin without much-affecting cellulose and generated 2.73 g/L of reducing sugars during hydrolysis (Wu et al. 2017). Ultrasonication is also a green approach and proved to be efficient at a laboratory scale. However, the main disadvantage is that it exhibits different efficacy toward a variety of lignocellulose biomass. Thus an optimized cost-effective technology development is still challenging. Moreover, it can be applied in combination with other methods for hybrid pretreatment of lignocellulose biomass.

2.3.3 Chemical Pretreatment Process

2.3.3.1 Ozonolysis

Ozone can be used as a potential oxidant to pretreat lignocellulosic biomass. It has the ability of delignification along with degradation of hemicellulose which leads to exposing the inner cellulosic core to the hydrolytic enzymes, accelerating the hydrolysis by 4–5 folds. Its powerful oxidant properties allow the breakdown and effective removal of lignin without producing any toxic by-products that may create inhibitory effects in downstream processes. As the process is operated in ambient conditions, no extra cost is required like other pretreatment methods which are carried out at high temperature and pressure. The enzymatic hydrolysis yield was increased from 29% to 88.6% after ozonolysis in wheat straw (García-Cubero et al. 2009). It was recently demonstrated that ozonolysis could effectively remove 20% lignin with 31% sugar yield through enzymatic hydrolysis from water-submerged municipal waste (Rosen et al. 2019). In another work, eucalyptus branches were subjected to ozonolysis with significant delignification (26.63%) and saccharification yield (68%) (Andersen et al. 2019). However, the high cost of ozone along with large requirements has limited its use on an industrial scale.

2.3.3.2 Acid Hydrolysis

Acid pretreatment of lignocellulosic biomass has been extensively used in industries. Strong acids like sulfuric, nitric, and hydrochloric acid are employed to remove lignin and hydrolyze the hemicellulose and cellulose for providing greater accessibility to the enzymes. Acid pretreatment can be performed in either concentrated acid at low temperature or a diluted acid at high temperature. The main advantages of this technique are the solubilization of hemicellulose and the precipitation of lignin at the same time. For example, acid pretreatment of coastal bermudagrass with 1.2% H_2SO_4 at 140 °C for 30 min retention time gave rise to a total sugar yield of 94% in the hydrolysis step (Redding et al. 2011). However, during the process, the degradation of monosaccharides leads to the formation of many unwanted inhibitory by-products such as 5-hydroxymethylfurfural (HMF) and 2-furfuraldehyde (Saha 2004). Moreover, high-temperature treatments sometimes produce furfural which gets degraded into formic or levulinic acids. These inhibitory compounds create a severe impact affecting the efficiency of the hydrolytic enzymes. Toxicity, acid recovery, and corrosiveness of the equipment are some of the considerable drawbacks of this approach. Current industrial processes are focused on reducing the inhibitory by-products by using diluted acid treatments.

2.3.3.3 Alkali Hydrolysis

Alkaline pretreatment is one of the common industrial methods for delignification. Alkaline agents like sodium hydroxide (NaOH), ammonium hydroxide (NH_4OH), calcium hydroxide ($Ca(OH)_2$), potassium hydroxide (KOH), etc. are used for pretreatment of lignocellulosic residues. Alkaline pretreatment induces solvation and saponification. These cause the deformation of complex networks of lignin, decrystallization of cellulose, cellulose swelling, and fractionation of lignin by breaking the ester linkages between lignin and other hemicelluloses in the complex network, thus promoting the solubilization of these components and enhancing the activity of the catalytic enzymes. For example, NaOH pretreatment (4–40 g/100 g dry straw) was found to decrease xylan content up to 46.37% and resulted in 64.55% glucose yield (Wan et al. 2011). Moreover, sodium carbonate (Na_2CO_3) pretreatment was found to yield significant total sugar (71.7%), glucan (76.1%), and xylan (73.2%) from rice straw (Yang et al. 2012). Alkaline pretreatment is very much effective for lignin removal and cellulose decrystallization and thereby increasing the enzyme accessible surface area. The energy requirement is also low due to the low process temperature. However, long residence time makes the neutralization step very difficult before the downstream and increases the overall cost of the process.

2.3.3.4 Organosolv Pretreatment

In this approach, lignocellulosic biomass is heated in the presence of organic liquids and a water mixture (ethanol-water, ethylene glycol, benzene-water, etc.). A significant amount of lignin and some amount of hemicellulose get dissolved in the process while the solid cellulose part is left in the medium. A wide range of organic compounds such as ethanol, methanol, glycerol, tetrahydrofurfuryl alcohol, dimethyl sulfoxide, ethers, ketone, etc. has been used to study the solvent extraction process (Thring et al. 1990). Pretreatment of wheat straw using aqueous glycerol has shown cellulose recovery of 95% along with 70% lignin (Sun and Chen 2007). For a purpose of utilizing whole lignocellulose biomass in biorefinery, ethanol organosolv pretreatment (50% (v/v) ethanol and 1% (w/v) sulfuric acid catalyst) was carried out with simultaneous generation of organosolv lignin (12 g), furfural (7.9 g), glucose (37.1 g), and hemicellulose-derived sugars (11.4 g) (Choi et al. 2019). In a recent study, hybrid Pennisetum (HP) biomass was pretreated with different organosolv (tetrahydrofurfuryl alcohol (THFA), γ-valerolactone (GVL), ethanol, and acetone) under 100 °C for 2 h which resulted in significant-high glucan yield (80.8%), and THFA was reported to be most effective among the organosolv for improved hydrolysis and lignin recovery (Tan et al. 2019). In another work, the use of ethanol-water resulted in 86% glucan and 62% lignin removal in *Eucalyptus nitens* bark (Romaní et al. 2019). This method is effective for the pretreatment of softwood materials with high lignin content and also for efficient fractionation of pure

cellulose, hemicellulose, and lignin. Organic solvents with a low boiling point can be easily recovered and recycled. However, solvents with high boiling points need high-pressure conditions, and the huge cost of the solvents makes the process expensive. The solvents must be well separated before the downstream process or else the growth of the fermentative microbes can be inhibited.

2.3.3.5 Oxidative Lime Pretreatment (OLP)

Lime is an effective oxidative agent for lignocellulose pretreatment for reducing the lignin content and enhancing better cellulose recovery. The efficiency of the delignification of thermal lime pretreatment can be greatly enhanced by introducing oxygen as an oxidant in the process. OLP effectively removes lignin and hemicellulose acetyl content from the biomass which makes the substrates more accessible to hydrolysis. Recent studies have shown that 68% and 98% of total xylan and glucan have been recovered, respectively, when switchgrass is pretreated using OLP at 110 °C and 6.89 bar for 240 min in combination with milling (Falls and Holtzapple 2011). In another study, lime pretreatment of corn stover resulted in 87.5% lignin removal along with a yield of 93.2% glucose and 79.5% xylose during the hydrolysis (Kim and Holtzapple 2005). One major disadvantage of this process is that a huge amount of water is required for washing out the calcium salts. Moreover, the cost of downstream processing also makes it expensive.

2.3.3.6 Ionic Liquid Treatment

The chemical pretreatments discussed above mainly consist of derivatizing solvents which cause chemical modification to the cellulose, reducing cellulose yield in the pure form. In recent years, Ionic Liquids (ILs) are considered a non-derivatizing solvent and have shown huge potential for the pretreatment of lignocellulosic biomass. ILs are eco-friendly and often referred to as "Green Solvents" (Wasserscheid and Keim 2000). The specialty of IL is that cellulose and lignin can be solubilized simultaneously. Ionic liquids are organic salts generally composed of a relatively large cation and a smaller anion, with a melting point of less than 100 °C (Rogers and Seddon 2003). IL has been proved to be a potential pretreatment agent due to its high thermal stability, low vapor pressure, and non-derivatizing solvent properties. Several ILs such as 1-ethyl-3-methylimidazolium-acetate ([EMIM][Ac]), 1-ethyl-3- methylimidazolium diethyl phosphate ([EMIM][DEP]), 1-allyl-3-methylimidazolium-chloride ([AMIM][Cl]), and 1-butyl-3-methylimidazolium chloride ([BMIM][Cl]) are some of the effective ILs to dissolve lignocellulosic biomass (Fig. 2.5) (Zhao et al. 2008). However, [EMIM][Ac] shows one of the best promising cellulose recovery among all the ionic liquids (Sun et al. 2009).

Pretreatment of wheat straw at 120 °C for 6 h using [EMIM][Ac] resulted in near 100% (w/w) sugar recovery (da Costa Lopes et al. 2013). [EMIM][Ac] along with buffer solution is used to successfully remove ~50% lignin from the wood chip

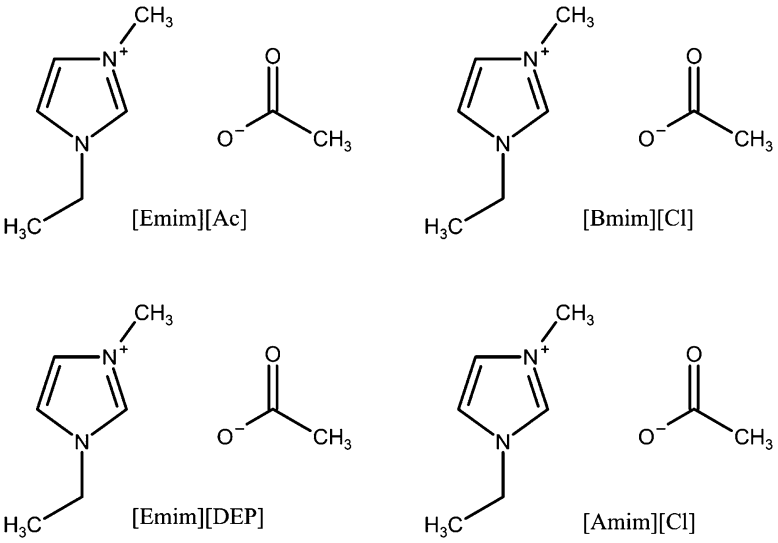

Fig. 2.5 Chemical structures of some imidazolium based ionic liquids are shown. 1-ethyl-3-methylimidazolium-acetate ([EMIM][Ac]), 1-butyl-3-methylimidazolium chloride ([BMIM][Cl]), 1-ethyl-3- methylimidazolium diethyl phosphate ([EMIM][DEP]), and 1-allyl-3-methylimidazolium-chloride ([AMIM][Cl])

biomass (Moniruzzaman and Ono 2012). To study the enzymatic saccharification, IL pretreated eucalyptus was subjected to enzymatic hydrolysis, and a nine-fold rise in cellulose conversion was observed (Uju et al. 2012). A high glucose yield of around 95.2% was found when the sugarcane bagasse was pretreated with IL and regenerated in water followed by enzymatic hydrolysis. Studies have been performed to optimize the process parameters of IL pretreatment which reported that dissolution can be enhanced by 50% using 67% aqueous-[EMIM][Ac] solvent mixture at 150 °C for 90 min, generating ~81% fermentable sugar (Fu and Mazza 2011). Several computational, as well as experimental studies, have been performed to understand the underlying mechanism of the process. The key factors governing the interaction between IL and lignocellulosic biomass are the cationic and anionic properties which vary with different ILs, the temperature, and the time of the pretreatment process. ^{13}C high-resolution NMR spectroscopy of cellulose oligomers in [BMIM][Cl] has revealed that the cellulose chains adopt specific conformational changes in IL environment (Moulthrop et al. 2005). Another study using ^{13}C and $^{35/37}Cl$ NMR relaxation in [BMIM][Cl] revealed that the hydroxyl protons of the cellulose chains interact with the Cl^- ions during the dissolution (Remsing et al. 2006). High temperature (~450 K similar to the industrial pretreatment temperature) vacuum molecular dynamics simulation study of crystalline I_β cellulose has shown a temperature-dependent behavior of cellulose microcrystal where significant deviation in the crystal unit cell parameters has been found due to the change in the rotamers of the hydroxymethyl group conformation (Bergenstråhle et al. 2007). This

change affects the stable hydrogen bonding network in the cellulose which facilitates the formation of hydrogen bonding between the anionic species of the IL and the hydroxyl group of cellulose chains. At the same time, the hydrophobic interaction occurs between the imidazolium ring of the cation and cellulose. These interactions break the stable inter- and intra-hydrogen bonds present within the lignocellulosic biomass, leading to the dissolution of the complex (Wahlström and Suurnäkki 2015). IL pretreatment increases the porosity of the biomass which makes it highly susceptible to enzymatic hydrolysis. Despite having so many attractive features of ILs, the main challenges of IL pretreatment are its high cost, recyclability, and recovery of ILs. To optimize the process, researchers are trying to use different binary mixtures along with IL during the pretreatment. In a recent study, it has been reported that the molecular behavior of [Emim][Ac]/water mixtures and its interaction with cellulose microcrystal reveals that 50–80% [EMIM][Ac] can be effective for both cellulose and lignin dissolution process (Manna et al. 2021; Manna and Ghosh 2019; Shi et al. 2014). Researchers have also used several organic solvents (N, N-dimethylacetamide, N,N-dimethylformamide, dimethyl sulfoxide, and pyridine) as diluents to improve the viscosity of IL for process optimization (Yang et al. 2019a). Progress has been made for a simultaneous pretreatment and hydrolysis process for cost-effective IL pretreatment technology (Husson et al. 2018).

2.4 Current Challenges in the Pretreatment Methods

At a process level, pretreatments are a multi-scale and non-homogeneous system where LCB interacts with the other components of physical and chemical agents. The complex heterogeneous nature of the LCB affects the overall reaction process leading to changes in yield in different pretreatment processes. This complexity has limited many of the traditional physical and chemical pretreatment processes in an experimental stage. Some of the key problems in pretreatment processes are given below:

1. Each pretreatment method has its own limitations. Some limitations of the traditional pretreatment methods are summarized in Table 2.3. However, a proper evaluation method needs to be developed in terms of economic feasibility and productivity to access the pretreatment processes.
2. The understanding of a physical or chemical pretreatment technology on the basis of the reaction mechanism is limited. More research needs to be done on the fundamentals to reveal the influence of particular pretreatment on the structure and digestion of cellulose and hemicellulose. The knowledge can be utilized to develop a suitable reaction model or optimization of the existing technologies.
3. Most of the pretreatment technologies primarily consider the cellulose hydrolysis, rate of hydrolysis, overall glucose yield, and lignin removal for the effectiveness of the process. However, it doesn't provide insight into the physical chemistry perspective in the reaction process. Thus, further, development is necessary.

Table 2.3 Overview of the challenges in some common physical and chemical pretreatment methods

Method	Challenges
Mechanical pretreatments	• Very expensive due to high energy input and operating costs • Actual performance varies with the type of LCB • Economically infeasible compared to other pretreatment methods
Wet oxidation	• Overall hydrolysis yield is lower than yields obtained in the steam pretreatment
Liquid hot water	• Water consumption and energy inputs are very high • Selection of the appropriate temperature is important
Ozonolysis	• Ozone generation and supply are crucial • High-operation cost
Steam explosion	• Incomplete deconstruction of LCB leads to precipitation of lignin components making it less digestible by enzymes. Also, some amount of xylan degraded and lost • Generation of inhibitors at higher temperatures • Loss of soluble sugars due to extensive washing
Super critical CO_2	• Less effectiveness of pretreatment • Capital cost is very high due to operational conditions with high pressures and energy input for carbon dioxide liquefaction
Acid pretreatment	• The amount of water required for washing is very high • Formation of enzyme inhibitors • Corrosiveness of the acid affects the reactor lifespan
IL	• High cost of IL • Recycling of the IL needs to be optimized • Inhibitory effects against hydrolytic enzymes
AFEX	• High cost of ammonia • High equipment maintenance cost • The corrosive properties affect the reactor
Organosolv	• Requirement of high process pressure • Cost of organic solvents and their recycling are high • Controlled environment is required due to explosion hazards and environmental concerns

4. Even if some methods are effective to the laboratory scale, the scale-up becomes more challenging due to the high cost, the requirement of optimal process conditions, and involved environmental concerns.

2.5 Conclusion

Pretreatment is the key step in the deconstruction of LCB for producing any biofuel or value-added chemicals. The effects of the traditional pretreatment methods on the different components of LCB have been discussed in detail. The crystallinity of the cellulose and removal of lignin are crucial for the effective enzymatic hydrolysis and overall sugar yield. Though few of the processes can help significant yield, the scale-

up for industrial application is still a challenge to overcome. The challenge can be addressed in two ways. Either the existing techniques should be improved, or else research should be done on developing new eco-friendly, cost-effective, efficient pretreatment methods. Besides, an investigation needs to be done on LCB components to design efficient separation methods. Moreover, when a single pretreatment technique is used, it raises various technological drawbacks, environmental concerns, high-operation costs, and compromised productivity. Thus, the initiative should be taken to combine multiple pretreatment methods including physical and chemical processes. For example, mechanical crushing–electronic radiation–alkali treatment, mechanical crushing–microwave–chemical processing, microwave-IL treatment, mechanical crushing–chemical treatment–steam explosion, and others. By means of a combined pretreatment method, the deconstruction of lignocellulosic biomass can be significantly improved in terms of accessible surface area to the enzyme, efficient hydrolysis, and production of biofuel and value-added chemicals in a cost-effective manner.

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Chapter 3
Physical and Chemical Hydrolysis Methods for Breaking Down the Complex Waste Biomass to the Fermentable Sugars and Value-Added Products

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Abstract Agricultural, industrial, and household practices generate a wide variety of waste biomass that is generally underutilized and often contributes significantly to environmental pollution. Such waste streams are rich in complex polysaccharides (cellulose, hemicellulose, and pectin), proteins, and lipids that can be hydrolyzed into fermentable sugars (hexoses and pentoses) or other added-value products (peptides, fatty acids, organic acids, carotenoids, etc.). However, the conversion of complex polymeric substrates into fermentable sugars is carried out by means of various physical and chemical methods. Physical methods of biomass treatment such as grinding, milling, microwave radiation, and ultrasonication are primarily aimed at reducing the size of the structural biopolymers and exposing the lignocelluloses to chemical reagents or enzymes for further hydrolysis. Conventionally, acid or alkali is used for hydrolysis of pretreated lignocellulosic biomass (such as agro and forestry residues). Other methods of physicochemical treatment such as liquid hot water treatment, autoclaving, or ammonia fiber expansion can be selected depending upon the biomass characteristics. Similarly, wastewater rich in proteins or lipids from industries such as dairy, oil refineries, and poultry is traditionally treated with

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hydrolytic enzymes (proteases and lipases) prior to anaerobic biodegradation. This chapter provides a comprehensive review of the various physical, chemical, and physicochemical methods for a breakdown of complex polymeric substrates in the waste streams into either simpler fermentable sugars or other bioproducts of commercial value.

Keywords Hydrolysis · Lignocellulosic biomass · Wastewater · Chemical methods · Physical methods · Hydrolytic enzymes

Abbreviations

CAS	Conventional active sludge
COD_p	Chemical oxygen demand
DES	Deep eutectic solvent
FW	Food waste
P&P	Pulp and paper
POM	Particulate organic matter
TS	Total solid
TSS	Total soluble solids
WCO	Waste cooking oil
WWTP	Wastewater treatment plant

3.1 Introduction

We commonly overlook waste products including food waste (FW) in our daily life. However, lately waste food materials are receiving much interest among a varied group of scientists. Globally, FW is recognized as a major problem, the waste generated has a considerable impact on society and the environment. Thus, managing FW poses a challenge. Conventionally, the FW is either dumped in landfills or incinerated posing a serious threat to the environment as well as human health, in turn causing huge economic losses. Currently, conversion into value-added products has emerged as a potential solution in managing agricultural and food waste. Largely, the agricultural and food are composed of carbohydrates (simple sugars and complex polysaccharides), proteins, lipids, along with bio-pigments and bioactive constituents, which is quite evident. Thus, wastes with such important constituents can be converted to value-added products such as phytochemicals, dietary fibers, natural colorants, and livestock feed to name a few which can play an important role in food waste management.

Bioplastics are a highly valued product that can be produced using FW. However, such conversion requires pretreatment of waste to generate monomers such as sugars that can be later converted into bioplastics by varied processes. Both acid and alkali

pretreatment help in solvation of the solid waste, i.e., depolymerization of plant polysaccharides and lignin. Also, the involvement of physical methods such as ultrasonication and heating can further improve the depolymerization process. On the other hand, using enzymes or microbial fermentation can also be efficiently utilized as pretreatment methods (Tsang et al. 2019; Bhardwaj et al. 2021; Bhardwaj and Verma 2021). Physical treatment such as heating combined with acid treatment has shown promising results in converting FW streams to generate monomers like 3-hydroxyvaleric acid which can later be polymerized into bioplastics (Ahn et al. 2016).

Alternative fuels are highly sought-after products these days, and the value of energy generated from the fuel is well recognized by all energy-intensive sectors. Food wastes specifically waste cooking oil (WCO) or grease coming out from food outlets have been shown to yield biodiesel through an alkali catalyzed transesterification reaction with alcohols. Importantly, WCOs are considered economical sources for fuel production. Methanol is the most commonly used alcohol that has been utilized for efficient transesterification reactions in presence of catalysts such as KOH or NaOH or CaO, leading to sufficient yield in biofuels. The prepared biofuels can be blended with conventional fuels to run compression ignition engines (Singh et al. 2021), and also the combustion values were within the range mentioned by American Biodiesel Standards. Researchers have met quite a success in such reactions on used cooking oils such as palm oil, canola oil, peanut oil, olive oil, or cooking oil mixtures (Sahar et al. 2018; Degfie et al. 2019; Park et al. 2019).

Emulsifiers are quite important ingredients used in the food industry specifically to produce stable oil-in-water emulsions. Emulsifiers can be extracted from food waste through both physical and chemical processes. On characterization, certain emulsifiers have been found to be made up of proteinic and poly/oligosaccharide materials. Researchers have extracted emulsifiers from FW such as winery wastes, olive mill waste or compost, etc. (Koliastasi et al. 2019).

Again, special value-added products such as ploy phenolic compounds, antioxidants, and colorants have been identified as important components of fruits and vegetable wastes such as peel, pomace, and seeds. Resveratrol is a highly valued polyphenol with potent bioactivity and has been extracted at an industrial scale using supercritical CO_2 extraction methodology from grape pomace as a waste of the wine industry (Casas et al. 2010; Saini et al. 2021). Again, the deep eutectic solvent (DES) extraction method can significantly recover resveratrol from peanut roots (Chen et al. 2018). Carotenoids are another prominent component and are widely regarded as a natural food colorant (Goswami et al. 2021a, b; Mehariya et al. 2021). Luengo et al. (2014) suggested improved extraction of carotenoids by subjecting tomato waste to ultrasonication. Also, comprehensive reviews have suggested the employment of varied physical and chemical methods for the extraction of specialty constituents. For instance, physical methods such as high pressure and temperature treatment and ultrasound-assisted extraction with organic solvents like ethanol have assisted in the extraction of phenolic acids and flavonoids from grape skin onion and tobacco wastes. Again, microwave-assisted extraction of pectin (a soluble dietary fiber)

from the peel of citrus fruits peel and passion fruit peels also improves the extraction process and yields pectin with similar characteristics to that of industrial pectin (Yukesh Kannah et al. 2020). Also, lycopene has been extracted from dry tomato peels using supercritical CO_2 extraction as well as with solvents such as hexane, ethyl acetate, and ethanol (Kehili et al. 2017). Astaxanthin (a pigment) from pink shrimp waste can be recovered using water-based ultrasound treatment (da Silva et al. 2018). Many of these valued products are also a part of non-nutritive but important health-benefiting constituents as they have potent bioactivities and can be added to food products to develop functional foods (Saikia et al. 2016, 2020). Other valued products for the industry such as organic acids (citric, lactic, succinic, propionic acids, etc.), enzymes (tannase, lactase, alpha-amylases, etc.) are mostly produced using microbial fermentation (solid-state/submerged state) with the help of varied microbial strains such as *Aspergillus niger, Actinobacillus succinogenes, Lactobacillus, Actinobacillus succinogenes, Bacillus licheniformis, Rhizopus, Thermomyces lanuginosus* or their recombinants. Compilation of numerous studies has suggested the use of FW such as fruit pomace and peels, corn cobs, coffee mucilage, lipid-rich wastes, etc. for production (Verma and Shah 2022; Verma 2022). However, there is lack of studies pertaining to mixed food waste as a medium for the generation of such acids or enzymes (Merrylin et al. 2020). On the other hand, postproduction, techniques like dialysis, diethylaminoethyl (DEAE) cellulose, ultrasound-assisted enzymatic extraction, ion-exchange chromatography, and size-exclusion chromatography are commonly used for extraction, purification recovery of such valued products. Pretreatment or extraction using ultrasonication, supercritical CO_2 extraction, solvent extraction, pulsed electric field, etc., have also been employed to recover pigments, essential oils, and other aromatic esters from varied FW sources typically essentials oils from fruit/vegetable wastes (Sharmila et al. 2020; Devi et al. 2020). Overall, one can suitably understand that FWs can be effectively manipulated through various physical, chemical, or biological interventions and be an impactful source of varied valued constituents of use.

3.2 Physical and Chemical Hydrolysis Methods of Waste

Human agricultural activities have led to the production of large amounts of agricultural waste which are underutilized and dumped as litter to rot, polluting the environment (Obot et al. 2008). Large amounts of biomass accumulating annually not only deteriorates the environment but also reduces the availability of materials having an enormous potential value that can be transformed into a range of useful added products, comprising food (groundnut, cottonseed), fuel (ethanol, biogas), feed (by-products of sugarcane, sugar beets), and a variety of chemicals (pesticides, insecticides). Farming economies are rendered intricate due to mismanagement of agro-industrial residues, and these remnants are considered to be the most ample and renewable resources on earth. These agro-industrial wastes comprise cellulose (organic compound composed of β glucose units), hemicellulose (xyloglucans,

mannans), lignin (complex polymer comprised of aromatic alcohols known as monolignols), and other precipitates (Ghose 1956; Aberuagba 1997; Agrawal and Verma 2020; Kumar and Verma 2020a). Amid all, cellulose consists of the largest percentage which is typically found in the plant cell wall. Considering that hemicellulose and cellulose form the cell wall constituent and are lignified; therefore, there is a growing need for an efficient and inexpensive method of separating them from the cell wall. Furthermore, cellulose can be hydrolyzed for human consumption to yield glucose, which can be used as a substrate for the fermentation of valuable chemicals, such as alcohols and other organic compounds (John et al. 2007; Qi et al. 2009; Kumar and Verma 2020b). Considering the above facts, physical and chemical treatments alone or in combination may be utilized as a hydrolytic procedure to break down the wastes resulting from the agro-industrial process.

3.2.1 Physical Treatment Methods

It involves mechanical crushing, microwave treatment, ultrasonic treatment, and high-energy electron radiation methods.

3.2.1.1 Mechanical Crushing

This method comprises dry crushing (drying followed by crushing of the material), wet crushing (milling process carried out in water or liquid), and vibrating ball mill grinding (particles of the materials are crushed between porcelain or metal balls and the mill body), and compression. It often precedes other operations to make succeeding procedures easier and more efficient.

Mechanical splintering can be used to reduce the particle size of lignocellulosic feedstocks to increase the surface area that is exposed to subsequent acids or enzymes. However, the high cost associated with this procedure along with its inability to efficiently remove lignin and hemicellulose from the cell wall constituent, limits its suitability (Hendriks and Zeeman 2009; Hu et al. 2014; Jin et al. 2015; Nelson et al. 2013).

3.2.1.2 Microwave Treatment

Typically, the radiation of microwave is considered the most usual method for the treatment of plant biomass. In addition to ease of pretreatment, increasing the capacity of heating, reduced time processing, minimal generation of inhibitors, and lower energy consumption, this pretreatment method has various advantages The microwave treatment enhances the adjustability of lignocellulosic raw material to enzymes, subsequently increasing the enzyme's activity effect (Chen et al. 2017a, b; Kumar et al. 2020).

3.2.1.3 Ultrasonication

In addition to opening crystalline cellulose, ultrasound can dissolve lignin molecules and markedly improve cellulose's accessibility and chemical reactivity. Nevertheless, it has only a minor impact on cellulose's fine structure. Hemicellulose can undergo disintegration when treated with ultrasounds. This results in a decreased fiber-to-surface area ratio, which in turn interferes with the enzymatic hydrolysis process. Cavitation effects, which trigger high enzymatic hydrolysis, are a result of using ultrasonic waves in enzyme processing (Chen et al. 2017a, b).

3.2.2 Chemical Treatments

Chemical hydrolysis procedures comprise acid/alkali treatments (Kiyoshi et al. 2015; Seifollahi and Amiri 2020).

3.2.2.1 Acid Pretreatment

Acid hydrolysis is a preferred and considered technique as it is relatively quick and inexpensive for hydrolyzing the cellulosic biomass generated as a part of agricultural waste (Palmqvist and Hagerdal 2000; Megawati et al. 2010; Bhardwaj et al. 2020). Temperature (120–180 °C), acid concentration (high or low strength), a total solid fraction (TS), and time duration (varying from minute to an hour) are the key parameters that affect the cellulosic and lignocellulosic biomass during acid hydrolysis (Grohmann et al. 1995; Talebnia et al. 2007). The glycosidic bonds are formed between hemicellulose and cellulose, and its susceptibility to acid forms the basis of acid pretreatment. Both inorganic acids such as sulfuric acid (Kärcher et al. 2015), phosphoric acid (Nair et al. 2015), nitric acid (Kim et al. 2015), and hydrochloric acid (Zu et al. 2014) and organic acids such as formic acid (Du et al. 2016), maleic acid (Jung et al. 2015), and oxalic acid (Jeong and Lee 2016) are used. Additionally, inhibition by compounds such as furfurals, 5-hydroxy methyl furfural, phenolic acids, and aldehydes formed as a result of undesired cellulose degradation during acid treatment makes it less attractive as a pretreatment option.

3.2.2.2 Alkali Treatment

The solubility of lignin in the alkali solution is crucial to the alkali pretreatment process. Alkaline pretreatment of lignocellulose can be done with NaOH, KOH, Ca $(OH)_2$, and ammonium hydroxide. During the alkali pretreatment procedure, a saponification reaction occurs, causing the intermolecular ester bonds between hemicelluloses and lignin to be cleaved. This causes lignin and hemicellulose

particles to dissolve in the alkali solution, allowing enzymes to interact with the cellulose (Li et al. 2016). Furthermore, alkali pretreatment alters the lignocellulosic structure by causing cellulose swelling, which reduces crystallinity and degree of polymerization, resulting in an increase in internal surface area (Behera et al. 2014). In addition, alkali pretreatment enhances the accessibility of carbohydrates to enzymatic hydrolysis by removing acetyl groups and uronic acid substitutions in hemicelluloses (Maurya et al. 2015).

3.2.3 Enzymatic Treatment

The use of biocatalysts, such as enzymes like lipase and cellulase, plays a vital role in the hydrolysis and breakage of polymeric chains present in carbohydrate-rich feedstocks (Binhayeeding et al. 2020; Koti et al. 2016; Tavva et al. 2016).

3.3 Conventional Chemical Hydrolysis Methods for the Treatment of Agricultural Wastes

Crop and livestock farming generates a large volume of agricultural residues that pose a serious environmental concern when it is not treated or managed well. Crop residues are primarily generated from the cultivation of cereals, oil crops, pulses, roots and tubers, and fiber crops. These residues are rich in polysaccharides such as cellulose, hemicellulose, and pectin that can be converted into monomeric sugars such as glucose and xylose, and sugar acid (D-galacturonic acid) by the process of hydrolysis. However, a prior pretreatment step is necessary to remove the lignin and make cellulose more accessible to the hydrolytic enzymes. Conventionally, acid-alkali pretreatment followed by enzymatic hydrolysis has been used to treat a wide variety of agro-residues such as palm empty fruit branch, barley straw, sugarcane bagasse, and different grass types (Martínez et al. 2015). Alkali pretreatment has been reported to remove lignin extensively that resulted in more than 90% conversion of glucan into glucose by enzymes (Martínez et al. 2015). Of late, the efficiency of enzymatic hydrolysis has been further improved by combined pretreatment approaches. For example, in the case of rapeseed straw, delignification by hydrothermal dilute acid pretreatment followed by alkali posttreatment resulted in a significant increase in hydrolysis rate by 5.9 times (Chen et al. 2017a, b). Similarly, sequential dilute acid-alkali/ammonia treatment resulted in increased enzymatic hydrolysis of poplar biomass (Shi et al. 2020).

Although enzymatic saccharification is a widely used method for the conversion of structural carbohydrates present in the pretreated biomass into monomeric sugar, the high cost of the enzymes remains a major bottleneck. In this regard, the role of renewable heterogeneous catalysts in the breakdown and conversion of

Table 3.1 Different chemical hydrolysis methods of wastes for the production of value-added products

Pretreated agro-residues	Hydrolysis method	Conversion/ saccharification efficiency (%)	Product	Reference
Barley straw	Commercial cellulases (Cellic® CTec2)	91.19 g/L (82% hydrolysis yield)	Fermentable sugars and ethanol	Paschos et al. (2020)
Rice straw, corncob, sugarcane bagasse, and banana stem	Hydrolytic enzymes from *Aspergillus tubingensis* are produced using black liquor	745.50 (86.02), 596 (74.5), 358.15 (42.98), 245.70 (33) mg/g of TRS, respectively	Reducing sugars	Narra et al. (2020)
Potato peel waste	Commercial enzymes (cellulase and amylase) or (alpha-amylase from *Bacillus* sp. Gb67) used either separately or in mixtures	72.38% (for commercial enzymes)	Fermentable sugars and ethanol	Atitallah et al. (2019)
Mixed food and beverage waste	Glucoamylase and sucrase	Glucose (228.1 g L^{-1}) and fructose (55.7 g L^{-1}), 0.17 g sugar per g mixed waste	Sugar rich hydrolysate	Kwan et al. (2018)
Date palm cellulosic wastes	Cellulases from *Geobacillus stearothermophilus*	31.56 mg/mL of glucose	Fermentable sugars and lactic acid	Alrumman (2016)
Corncob	Rice husk-based solid acid catalyst	TRS yield of 486.53 mg/g and xylose of 253.03 mg/g	Fermentable sugars (xylose, arabinose, glucose)	Chen et al. (2019a, b)
Mixed rice straw and de-oiled algal residue	Garlic peels derived solid acid catalyst	41.41%	Fermentable sugars, lipids, and carotenoids	Bardhan et al. (2022)
Rice straw (RS)	Glycerol-based solid carbon acid catalyst	262 mg/g TRS (from alkali pretreated RS) 147 mg/g TRS (from native RS)	Fermentable sugars and ethanol	Goswami et al. (2015)

lignocellulosic biomass is an emerging area of interest (Lin et al. 2021). Table 3.1 below depicts the saccharification of agro-residues using different chemical hydrolysis methods for the production of biofuels and other value-added products.

3.4 Conventional Hydrolysis Methods for Treatment of Poultry and Dairy Wastewater

Besides crop residues, the agro-industrial sector also generates a wide range of various waste streams such as poultry manure, animal wastes, and wastewater from the dairy processing industries. These wastes are rich in organic matter with a high carbon content, while the macronutrient (N, P, K) content varies considerably depending on the type of the feedstock (Markou and Monlau 2019). However, the wastes/wastewater have to be pretreated and hydrolyzed before the organic carbon is readily available for microalgal growth or anaerobic digestion for the production of suitable bioproducts. Usually, the wastewater from poultry slaughter-houses or dairy industries is rich in lipids and proteins. Enzymatic hydrolysis (commercial lipases) was used to convert the fats in the flotation froth (hydrophobic material phase-separated from wastewater by flotation) into long-chain free fatty acids during anaerobic digestion (Pascale et al. 2019). In another study, 10% garbage enzyme (obtained by the fermentation of fruit or vegetable peels, molasses, and water) was used to treat synthetic dairy wastewater and was found to reduce total soluble solids (TSS) by 26% at pH 6.5 after 5 days of treatment (Sambaraju and Lakshmi 2020). Researchers have also reported the application of whole-cell catalysts such as *Penicillium citrinum* whole cells in decreasing organic matter and lipid content in dairy wastewater. In the same study, it was reported that free whole cells achieved higher hydrolysis efficiency (92.5%) as compared to immobilized whole cells (Alves et al. 2019). Although enzymatic hydrolysis (using lipases, protease, keratinase) is conventionally used for the conversion of lipids and proteins into fatty acids and peptides/amino acids, respectively, certain new promising technologies have been developed in recent years for recovering value-added products from the food processing industry wastes. One such tool is the "sub-critical water hydrolysis" method finding wide application in the recovery of protein hydrolysates from animal and vegetable wastes (Marcet et al. 2016).

3.5 Conventional and Recent Hydrolysis Methods for the Treatment of Sewage and Sludge

Sewage from both domestic and municipal sources consists of organic and particulate matters, thus regarded as complex wastewaters. Organic polymers, viz., carbohydrates, proteins, and lipids constitute about 30–70% of particulate chemical oxygen demand (COD_P) of domestic sewage. These organic particulate matters are reported to hinder or decrease the efficiency of wastewater treatment processes (Rajagopal et al. 2019). Wastewater treatments are high-energy driven and usually expensive processes. High-energy consumption and utilization of various resources are the main disadvantages of conventional wastewater treatment methods. It has been reported that about 3–4% of total U.S. electricity is utilized in the wastewater

treatment process and such is the case with other developed and developing nations. Additionally, high-energy consumption is associated with the production of a large amount of carbon into the atmosphere which could be responsible for enhanced global warming (U.S. Environmental Protection Agency 2006). The increase in population and the subsequent release of excessive wastewaters into the environment have put a burden on social sustainability and stability due to the water-energy inefficient wastewater treatment methods. The conventional active sludge process (CAS) for wastewater treatment requires extensive energy-driven aeration and thus is not recommended as an energy-efficient method. In a CAS process, it has been estimated that about 50% of the total energy consumption is utilized for supplying air to the aeration tanks only (Waqas et al. 2020). Therefore, energy-efficient wastewater treatment methods are required to decrease the burden on energy resources and maintain economic stability as well as social sustainability. In order to avoid the limitations, hydrolysis has been applied as an important part of anaerobic treatments. Hydrolysis (biological pretreatment) involves the breakdown of complex organic molecules into simpler ones by hydrolytic enzymes secreted by fermenting bacteria. The applications of hydrolysis include partial removal of COD by fermentation and an increase in biodegradability (Zhang et al. 2021). As hydrolysis is based on anaerobes and supply of aeration and external temperature is not required, therefore employed as energy and cost-efficient method for large scale wastewater treatment. The study carried out by Bian et al. (2018) demonstrated the effect of COD load on hydrolysis in a pilot-scale hydrolysis-aerobic system and found that 39–47% COD removal was achieved even at a COD load of 1.10 kg/m^3·day in municipal wastewater. It has been documented that 50% of the organic load (BOD/COD (kg/m^3 day)) entering the municipal wastewater treatment plant (WWTP) is comprised mainly of particulate organic matter (POM). Therefore, it is a prerequisite that POMs should be broken into smaller pieces through hydrolysis before their treatment and conversion to valuable by-products. Alvarado et al. (2021) studied the hydrolysis of particulate organic matter in municipal wastewaters under aerobic conditions. They found that during the first days, the high molecular weight of organic molecules increased and bacteria produced sufficient enzymes for their further degradation. On the other hand, they also found that oxygen utilization rate (OUR) developed continuously indicating the conversion of less-biodegradable organic matter to easily biodegradable organic matter.

It is evident that with improved wastewater treatment methods production of sludge has also increased tremendously throughout the world. The sludge is enriched in not only organic matter but also contains various toxic substances including heavy metals, persistent organic pollutants, and a wide variety of pathogens posing a threat to the environment. Therefore, emphasis is put on reusing valuable components of the sludge and energy recovery by different physicochemical methods particularly anaerobic digestion (Xu and Lancaster 2009). Organic matter is inaccessible to bacteria due to its complex nature; therefore, enzymatic hydrolysis is required to increase their bioavailability. It has been demonstrated that enzymatic hydrolysis improved sludge solubilization and acidification. Yu et al. (2013) studied the effect of endogenous amylase, protease, and combined amylase-protease treatment on

sludge anaerobic digestion. They found that combined amylase-protease treatment enhanced biodegradability along with the 23.1% increase in biogas production. It is important to discuss that hydrolysis also enhances the reduction of sludge production as well as the dryness and compactness of sludge cakes. Neyens et al. (2003) reported that hot acid hydrolysis reduced the sludge volume production by 70% more than the initial volume and the DS-solid content of the dewatered cake increased twice the initial untreated value. Hydrolysis treatment is significant for treating wastewaters from dairy industries with high protein and fat content. The study carried out by Mendes et al. (2006) reported that 12 h hydrolysis pretreatment of lipid-rich wastewater from dairy industries resulted in the enhanced levels of production of biogas (445 $\pm$ 29 mL) and organic matter (78.2%) as well as color removal. As discussed, wastewater sludge is a by-product of wastewater treatment processes and has become a major environmental problem for metropolitan cities and towns due to its huge production, high disposal cost, etc. The current approaches employed for sludge management and disposal include anaerobic digestion, landfill, and incineration (Zhang et al. 2017). Expensive sludge treatment and disposal, poor dewaterability, and inefficient utilization of wastewater sludge are the main challenges for wastewater sludge management (Chen et al. 2019a, b). The wastewater consists of about 40%, 14%, and 10%–25% proteins, carbohydrates, and lipids, respectively, but the conventional methods of wastewater sludge treatment could not recover an ample amount of organic and nutrient resources(Youssef et al. 2011). In this regard, thermal hydrolysis is gaining interest as an efficient method for wastewater sludge treatment due to its efficient sludge reduction as well as enhanced sludge dewaterability (Zhao et al. 2013). Liang et al. (2021) developed a three-phase sludge treatment and reduction method by incorporating thermal hydrolysis followed by fungal fermentation and subsequent anaerobic digestion. It was found that temperature treatment (140–180 °C) significantly reduced sludge volume while organic release efficiency was enhanced. The sludge liquor obtained at 160 °C consisting of the highest concentrations of carbohydrates and proteins was subjected to fungal fermentation by *Aspergillus niger* promoted the conversion of waste organics into valuable fiber materials. It was also demonstrated that the obtained fungal hyphae could be developed for making papers or some other value-added fibrous products. Therefore, the integration of thermal hydrolysis with fungal fermentation turned out to be an effective sludge reduction and waste organic valorization approach. As we know enzymatic hydrolysis enhances the biodegradability of sewage and sludge; therefore, its pretreatment with commercially available enzyme or inoculation of in situ enzyme-producing bacteria could promote biodegradation and production of by-products. The study carried out by Agabo-Garcia et al. (2019) demonstrated the application of commercially available proteases as well as the inoculation of *Bacillus licheniformis* as a pretreatment strategy to enhance the biodegradability and biogas production of raw sewage from aerobic digestor. The investigators found that biochemical treatments enhanced the stabilization and biodegradability of sewage sludge since the experiments showed higher depuration efficiency in terms of CODs (73–85%), TVS (30–42%), and CODt (16–28%) in comparison with the control experiment with CODs (38%), TVS (28%), and CODt

(12%). Fish industry discharges are often rich in total suspended solids (TSS), oil, and grease, and have high levels of COD as a result their release into the environment is quite harmful in terms of sustainability and stability. The inefficient conventional aerobic treatment of fish industry effluent has shifted the paradigm towards anaerobic treatments. Due to the excessive presence of oil and grease, the anaerobic treatments are hampered as the agglomeration and pellet formation hinders sludge sedimentation, thereby reducing treatment efficiency (Chowdhury et al. 2010). The enzymatic pre-hydrolysis treatment provides an alternative to reduce the oil and grease contents and speed up the treatment for fat-rich fish industry effluents. In this context, Duarte et al. (2015) demonstrated the enzymatic hydrolysis by lipase produced by the fungus *Penicillium simplicissimum* in solid-state fermentation and anaerobic treatment of fish processing effluent at different temperatures. They found that enzymatic hydrolysis at 50 °C and anaerobic fermentation at 30 °C promoted removal of 97.5% of chemical oxygen demand and the production of 105.4 mL CH_4/g $COD_{removed}$ was also achieved. It was suggested that thermophilic enzymatic hydrolysis reduced the overall amount of enzyme, hydrolysis time, and cost of anaerobic treatment, therefore could be applied on an industrial scale. In the pulp and paper (P&P) industry, the main area of interest is to reduce the biomass produced during wastewater treatment processes as sludge management accounts for 60% of total treatment cost (Mahmood and Elliott 2006). Due to the presence of various toxic substances and recalcitrance of P&P wastewater sludge as well as low production of methane, its anaerobic digestion is not as feasible as for domestic wastewater sludge. To achieve high feasibility of anaerobic digestion and enhance methane production during P&P wastewater sludge treatment, enzymatic pretreatment has been preferred as an important alternative. The enzymes decrease the complexity of biosludge, thereby enhancing anaerobic digestion with substantial enhancement in by-product production, methane yield, and/or COD solubilization (Yang et al. 2010). It has been reported that P&P sludge consists of 70% of proteins and carbohydrates; therefore, proteases, glycosidases, or combination are the desired candidates for pretreatment of P&P biosludge (Bayr et al. 2013). Bonilla et al. (2018) reported that proteases from *Bacillus licheniformis* enhanced the anaerobic digestibility of P&P biosludge and 26% increase in biogas production.

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

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Chapter 4
Critical Evaluation of the Role of Enzymes in the Integrated Biorefinery

Sumit H. Dhawane, Arka Ghosh, Piyush Dwivedi, Sneha Dey, Biswajit Kamila, and Gopinath Halder

Abstract Now-a-days, the rapid depletion of fossil fuels has created a global crisis of natural resources. Researchers are constantly focusing on the utilization of environment-friendly technologies and fuels. Integrated biorefinery possesses the ability to provide long-lasting, self-dependent, strong, environment-friendly alternatives for the production of various chemicals and biofuels. An integrated biorefinery is a modern idea derived from oil refineries that uses biomass to produce a plethora of products. The biorefinery can be divided into three major categories on the basis of biomass composition, namely triglycerides-protein based biomass, sugar and starchy, and lignocellulose. Enzymes have a great influence on biochemical processes in the transformation of carbohydrates and starch, and lignocellulosic biomass to bioproducts like biofuels and bioethanol. The optimization of various enzymes can be applied to different processes such as enzymatic hydrolysis and fermentation to increase the efficacy and maintain the stability of these enzymes used in bioprocesses.

Keywords Biorefinery · Biomass · Lignocellulosic biomass · Biorefinery processes · Optimization

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Abbreviations

ABE	Acetone Butanol Ethanol
CBM	Carbohydrate-Binding Module
CBP	Consolidated bioprocessing
CMC	Carboxymethyl cellulose
ED	Entner–Doudoroff
MOO	Multi-Objective Optimization
RSM	Response Surface Methodology
WS	Wheat straw

4.1 Introduction

Biorefineries have taken a great lead in the past 2 to 3 decennaries after the international oil crisis. So far, the western economies have commercialized biorefineries, and India is slowly coming up with that too. Integrated biorefineries are the same as the traditional refineries where every part of abundant biomass is viably converted into one of the useful products having the least wastage. This incorporates a whole worth chain for changing crude materials into stage particles, and their change results into biofuel. Biodiesel, bioethanol production, wood pulping, and anaerobic digestions are the prime processes in biorefineries (Cherubini 2010; Kumar et al. 2020)**.** Biomass in the refinery is mainly a mixture of hydrocarbons and releases more energy per gram in different steps in the biorefinery process. It can be transformed into a series of bioproducts in many ways like fermentation and thermochemical process. Ethanol and butanol are produced in the fermentation process. The thermochemical process involves the gasification process which decomposes biomass at large temperatures and pressure in the presence of a limited supply of oxygen for the production of synthesis gas and the pyrolysis process which also works under large temperatures in an almost oxygen-free environment (Kumar and Verma 2021). Bioforming is a chemo catalytic process that involves the formation of important carbohydrates from simple biomass at a mild temperature in an aqueous solution(van der Waal et al. 2013). Biomass principally comes from agricultural and crop wastes, forest crops, municipal waste, sewage, animal waste, etc. which acts as the raw material for biorefineries (Kumar and Verma 2020a).

In different industries, these raw materials are crops like wheat and maize, lignocellulosic crops, sugar crops (beet and cane), residues (stover and straw), etc. depending on the needs for product formation (Takkellapati et al. 2018; Balaman 2019; Singhvi and Gokhale 2019). In lignin-based biorefinery, a plethora of chemical bioproducts are produced like syngas (methanol, ethanol, Fischer-Tropsch liquids), hydrocarbons (benzene, styrene, biphenyls), and macromolecules (carbon fiber filters, polymer extenders, thermoset resins)(Holladay et al. 2007). In oleochemical industry, various biorefinery products are produced from C_{12} to C_{18}

saturated and monounsaturated fatty acids, like propylene glycol, epichlorohydrin, acrylic acid, and other glycerol derived chemicals (de Jong et al. 2012b). In a biomass, pyrolysis oil platform, an optimal combination of high and low-valued products can be derived from pyrolysis oil by thermal depolymerization of biomass at a controlled temperature (de Jong et al. 2012a).

Enzymes are an essential part of the biorefinery processes. Enzymes or biocatalysts are mixed with the substance in the same tanks where they are produced either as raw or as almost pure form or as a solution of different cocktails of enzymes. Biocatalysts have significant characteristics and can be used in biorefineries. Rather than that, they serve the application in different industries like biofuel, textile, paper-pulp, and brewing (Bhardwaj et al. 2019, 2021a, b; Kumar and Verma 2020a). Enzymes are recognized as alternatives to conventional synthetic chemicals as they pose a threat to the environment. Enzymes are derived from the plants-microorganism, and hence have no negative impact on the environment. Enzyme technology offers more advantages over conventional methods. This technology drives process yields and capacity and maximizes product quality and reduces the number of wastes (Nigam 2013). In oil and fat processing, mainly in the brewing industry, some enzymes provide continuous production capability and eliminate intermediate stabilizing agents which consume more energy and produce wastes (Gurung et al. 2013; Liu and Kokare 2017).

Enzymes play a major role in the conversion of biomass into different value-added products in the biorefinery processes (Agrawal and Verma 2020; Kumar and Verma 2020a). As the biorefinery industries grew, scientists started gaining interest in various enzymes involved in these.

Enzymes are biocatalysts that come from proteins (polymers of amino acids and small amounts of RNA) of animals and various microbes (Palmer and Bonner 2007). They have significantly taken down the power consumption and have produced more eco-friendly products with almost zero harmful wastes. Unlike typical catalysts, enzymes can react at normal temperature and pressure but an optimal temperature is maintained as it may rupture these enzymes and lead to a decrease in their catalytic activity (Punekar 2018). Biodiesel is produced from non-edible waste oil by some particular enzymes that convert fatty acids and triglycerides to desirable products having a high conversion rate(Ali et al. 2017). Conversion of structural polymers into monomers can be achieved by enzymatic fermentation using yeast or bacteria to produce biofuels.

Lipase is used as a catalyst in the esterification and transesterification process to produce methyl esters popularly known as biodiesel. Bacterial and fungal lipases are mostly preferred in the production of biodiesel. Nowadays, *Streptomyces sp.* is envisaged as a potent lipase-producing microbe for biodiesel production (Yücel et al. 2012). Monomeric sugars are produced by hemicellulose and cellulase hydrolyzing hemicellulose and cellulose. This sugar obtained from the pretreatment is now fermented using bacteria, yeast, etc. to produce ethanol.

The use of enzyme biorefinery processes reduces the price of final products. Laboratory-based research and constant development of this highly demanding process will remove these limitations in near future.

A large number of enzymes obtained from the microbial cells gained importance in the biorefineries for biomass transformation to final compounds. Thermostable enzymes entail various processes of biorefineries due to their efficacious solubility with the substrate, higher value of transfer rate, along with low contamination risk. A relatively stable enzyme can serve as a commercial catalyst also as it can endure the critical temperature conditions during the biorefinery processes (Anoopkumar et al. 2020). A meticulous study of the role of enzymes is further discussed in this article.

4.2 Different Processes and Products of Biorefinery

A biorefinery can be thought of as a system that handles and processes the raw biomass to give a plethora of products. The biomass associated with biorefineries may come from ranger service, farming, and hydroponics, and buildups from industry and families including wood, horticultural yields, and natural deposits.

For a century, the world has been vigorously depending on non-renewable energy sources like coal, oil, and flammable gas as energy assets, just as for petrochemical feedstock. Henceforth, various oil-based plants and refineries were being planned, built, and set up throughout the long term. Because of the different issues and difficulties emerging from the utilization of petroleum products and the expanding mindfulness of natural manageability, chemical industries are preparing to shift their interest from the exhaustible fossil fuel-based processes to more economical processes which depend on inexhaustible assets (Kumar and Verma 2020b; Goswami et al. 2021a, b). Among various kinds of renewables, biomass is perceived as one of the most encouraging assets to substitute petroleum products.

Biomasses originated from crops, algae or harvests buildups, lignocellulosic material, and municipal solid waste. Conversion of such materials into bioenergy and biomaterial in practice could be helpful in satisfying future market and industrial requests (Fig. 4.1). The transformation pathway of biomass is classified into biochemical, thermochemical, chemical, and mechanical processes. These process pathways can be consolidated into a biorefinery (Goswami et al. 2020; Kumar and Verma 2020b). The essential design of a biorefinery cycle and its items and results is displayed beneath:

4.2.1 Various Biorefinery Processes

The biorefinery processes incorporated for the production of various bioproducts from the natural biomass can be broadly classified into four major processes:

- *Mechanical processes*
- *Biochemical processes*

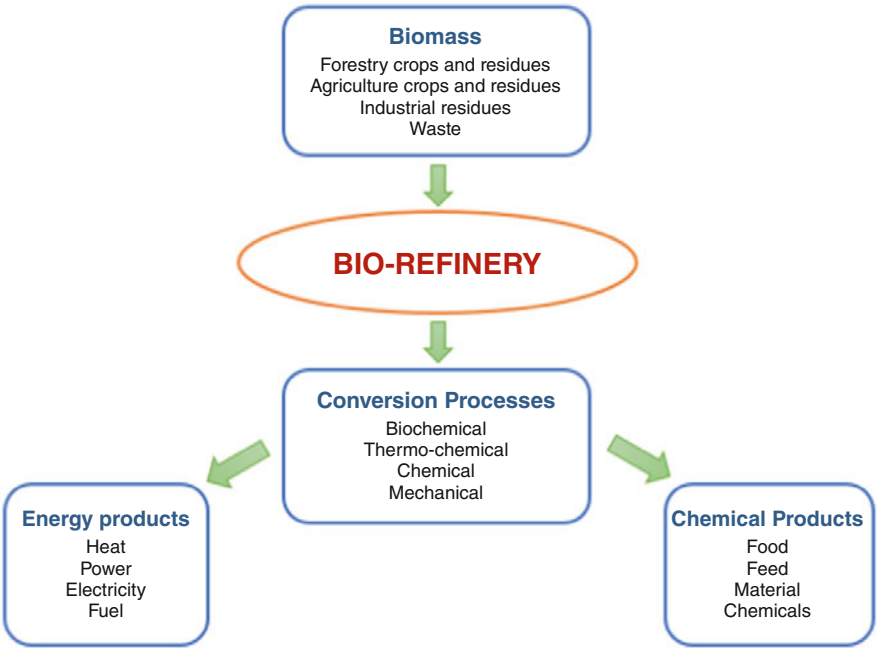

Fig. 4.1 General concept of a biorefinery

- *Chemical processes*
- *Thermochemical processes*

4.2.1.1 Mechanical Processes

The first and most significant advance in any conversion process is to lessen the actual size of biomass (Mosier et al. 2005). Mechanical processes don't change the state or the piece of biomass, however, just play out a size decrease of the feedstock. Decreasing the size of chips or fine powders enhances the mass and heat transfer which eventually results in the increment in the surface area of the concerned item (Fig. 4.2). The energy needed to diminish the biomass into a treatable size relies upon the density of the biomass source. Herbaceous materials don't need as much handling to accomplish the required molecule size as it does to lessen wood (Cadoche and López 1989).

Chipping is the most favored technique for wood reduction of size, as it reduces wood to 10–50 mm in 2D and 5–15 mm in 3D (Zhu and Pan 2010). This is the base treatment important to start conversion. Herbaceous biomass is obtained by forage cutting or shredding.

Along with chipping, various milling operations such as hammer milling, disk or attrition milling, and ball milling are visualized as some of the practical choices to

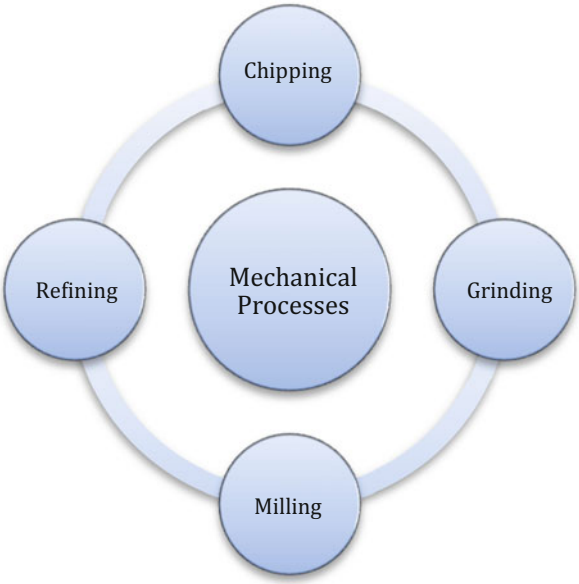

Fig. 4.2 Mechanical Pretreatment Processes

decrease biomass sizes. Hammer and disk milling operations are principally utilized in the production of wood flours and wood fibers creation on a huge scale for business purposes (approximately 1000 tones/day). Disk milling tasks are reliant upon ecological conditions and the nature of source materials. The energy necessity and the wood molecule size and shape rely upon these functional boundaries (Tienvieri et al. 1999).

Milling operations essentially affect downstream energy necessities and the productivity of enzymatic cellulose saccharification. Since the sole objective of a biorefinery is to improve the transformation cycle, with minimal energy usage and amplify the enzymatic cellulose saccharification, we have to take care of the biomass size. Failure at this stage intensifies the expense of energy necessities and lessens the adequacy of resulting therapies. Since these mechanical cycles can create a scope of molecule sizes, it is normally important to control the molecule size utilized in the biorefinery. Size characterization is refined utilizing strainers, screens, and imaging investigation. The molecule surface region is the most significant assurance of effectiveness, and consequently, it is the quality to be controlled (Holtzapple et al. 1989).

4.2.1.2 Chemical Processes

Chemical processes can be thought of as processes that convey an adjustment of the chemical structure of the concerned material during its reaction with discrete substances. Some of the well-known chemical processes incorporated for the conversion

of biomass are Fischer-Tropsch synthesis, transesterification, hydrolysis mechanization, etc. (Cherubini 2010).

The manufacture of stage chemical compounds through biotechnological fermentation has acquired huge consideration since it is a superior option in contrast to chemical synthesis, as it restricts the consumption of petrochemical assets, and diminishes ecological contamination by using sustainable biomass squanders (Wee et al. 2004).

The best strategy for bio-renewable amalgamation of chemicals is fermentation, which has mild temperature and pressure requirements, using the minimal expense of inexhaustible assets. Another advantage is the creation of minimal quantities of by-products (Pérez-Bibbins et al. 2013). A vital benefit of microbial fermentation is the creation of optically unadulterated mixtures in contrast with compound amalgamation, coming about in racemic combinations (Wang et al. 2010).

Another significant chemical process in biorefinery is the Fischer-Tropsch synthesis which that changes water gas into liquid hydrocarbons (De Klerk 2012).

The specific reactions involved in FTS are shown in the reactions numbered Eqs. (4.1), (4.2) and (4.3), respectively:

$$2H_2 + CO \rightarrow (CH_2) + H_2O \tag{4.1}$$

$$(2n + 1)H_2 + nCO \rightarrow C_nH_{2n+2} + nH_2O \tag{4.2}$$

$$(2n)H_2 + nCO \rightarrow C_nH_{2n} + nH_2O \tag{4.3}$$

The temperature and pressure requirements for this reaction are 150–300 °C and 1–10 atm, respectively, and the catalyst used is generally a metal catalyst.

These reactions happen in the presence of metal catalysts, ordinarily at temperatures of 150–300 °C (302–572 °F) and pressure of 1–10 atm.

The transesterification reaction of methanol and triglycerides is also a very vital process for biodiesel manufacture. This process is generally performed in an alkaline medium, or enzymes (lipase) acting as the catalyst in a temperature ranging about (323–35) K. Methanol is mostly preferred in the transesterification process.

4.2.1.3 Biochemical Processes

These are the processes with a low rate of reactions and low-temperature requirements. Two of the well-known biochemical processes are anaerobic absorption and fermentation. In the process of fermentation, various microorganisms are changed to form different products in the presence of additional enzymes. The most common products are natural acids and alcohol. As of now, ethanol is the most popular product of fermentation. Some of the hexoses, specifical glucose, are one of the most consecutive substrates of fermentation. On the other hand, pentose, i.e., hemicellulosic sugar, glycerol, and various other hydrocarbons stand in the need of the advancement of redid fermentation organic entities to facilitate their transformation into alcohols(Hamelinck et al. 2005). In anaerobic processing, there is a

bacterial disintegration of the biodegradable substance occurs. This process is carried out at a temperature of about 30–65 °C without any oxygen traces. Biogas is the principal final result of these conversion processes which contains methane, around 97% or more that can serve as a substitute in place of natural gas (Romano and Zhang 2008; Cherubini 2010).

4.2.1.4 Thermochemical Processes

In these refineries, fuel, synthetics, and energy are produced simultaneously. Thermochemical strategies for transformation incorporate pyrolysis ignition/burning, liquefaction, and gasification. Pyrolysis involves the change of organics over to strong, gas, and fluid by warming without oxygen. Measurement of the strong, fluid, and vaporous portions shaped are reliant on interaction factors. Burning is defined as the finished oxidation of some biomass. Likewise, incineration finds its application in manufacturing electricity. Here a tremendous measure of warmth is delivered and also off-gas is often utilized in running the turbine associated with the generator. The process of production of carbon dioxide, carbon monoxide, water, and hydrogen by incomplete oxidation is referred to as gasification. In liquefaction, water serves as the medium for the reaction and creates liquid hydrocarbons(Mohan et al. 2006).

The main prerequisites for any biorefinery are the feedstock characteristics and processes implemented so that the concerned products can be manufactured in a financially achievable way. The feedstock can be further divided into three major parts:

- First-generation feedstock
- Second-generation feedstock
- Third-generation feedstock

First-generation feedstocks are considered the consumable feedstock originating from the horticultural area, like wheat, corn, oilseeds, and sugarcane. These fundamental feedstocks are for the most part reaped with an excessive oil or sugar content, and changed to fuels, for example, biogas (combination of carbon dioxide and methane), biodiesels, and alcohols (Margeot et al. 2009; Srirangan et al. 2012).

Second-generation feedstocks are uneatable and include crude substances got from LCB and harvest squander buildups from different forestry and rural processes (Carere et al. 2008; Nigam and Singh 2011). These crudes are sometimes utilized for the production of synthetic substances and fuels. These feedstocks can be developed for a huge scope exclusively in the production of energy.

Third-generation feedstocks incorporate a huge assortment of photosynthetic algae. These algae have protein, sugar, and excessive lipid (approximately around 20–40% on a dry basis) or oil content. They can serve as an elective source of fuel because of their profoundly effective photosynthetic frameworks in sugar creation (Stamenković et al. 2011).

4.2.2 Various Products of Biorefinery

The various products from the various processes of the biorefineries are shown in Fig. 4.3.

4.3 Role of Various Enzymes

4.3.1 Methods of Enhancing Enzyme Efficiency and Stability

4.3.1.1 Roles of some Enzymes and Their Effectivity Improvements

Lignocellulose is a rich resource of sustainable carbon in the biosphere. Produced at a relatively high amount every year, it has an energy content almost comparable to 650 billion tons of raw petroleum, i.e., crude oils. Lignocellulose is made out of lignin, cellulose, and hemicelluloses. Based on the sources, cellulose and hemicellulose can be present around 40–50% and 25–30% of the complete composition individually (Yeoman et al. 2010). Consequently, hemicellulolytic expulsion gives better access of the enzymatic activity of cellulases to cellulose via decreasing the synergistic effects which reduces the requirement of addition of chemicals.

4.3.1.1.1 Cellulase

Cellulases and hemicellulases are used greatly in biomass processing in biorefineries by saccharification. Cellulose hydrolyzing glucanases, i.e., cellulases mainly include eta-1,4endoglucanase, beta-1,4exoglucanase, and beta-1,4 glucosidase. Both endoglucanases and exoglucanases are inhibited by their end product cellobiose and cellodextrins. Almost all cellulase strains have been divided into definite families, and almost the same chemical activities have been observed among some families (Zhang and Zhang 2013).

Cellulases are produced by varieties of microorganisms like fungus and bacteria. Some of them are *Trichoderma reesei*, *Clostridium thermocellum*, *Anaerocellum thermophilum,* and others (Escamilla-Alvarado et al. 2017). The effectiveness of reaction varies from species to species and even from the same species. These microorganisms utilize their discrete non-complexed cellulases and complexed cellulases as their daily source of nutrition. Glycoside hydrolases cleave glycosidic bonds using two residues of enzymes: a proton donor and a nucleophilic base. Depending on the structural arrangements of these residues, hydrolysis may occur via retention or inversion of anomeric configurations.

Endoglucanase belongs to the family of the glucanases that hydrolyze polymers such as glucans, laminarin and produce glucose as an end product. Endoglucanases or CMCase mainly depolymerize cellulose like beta-1,4 endoglucanase breaking

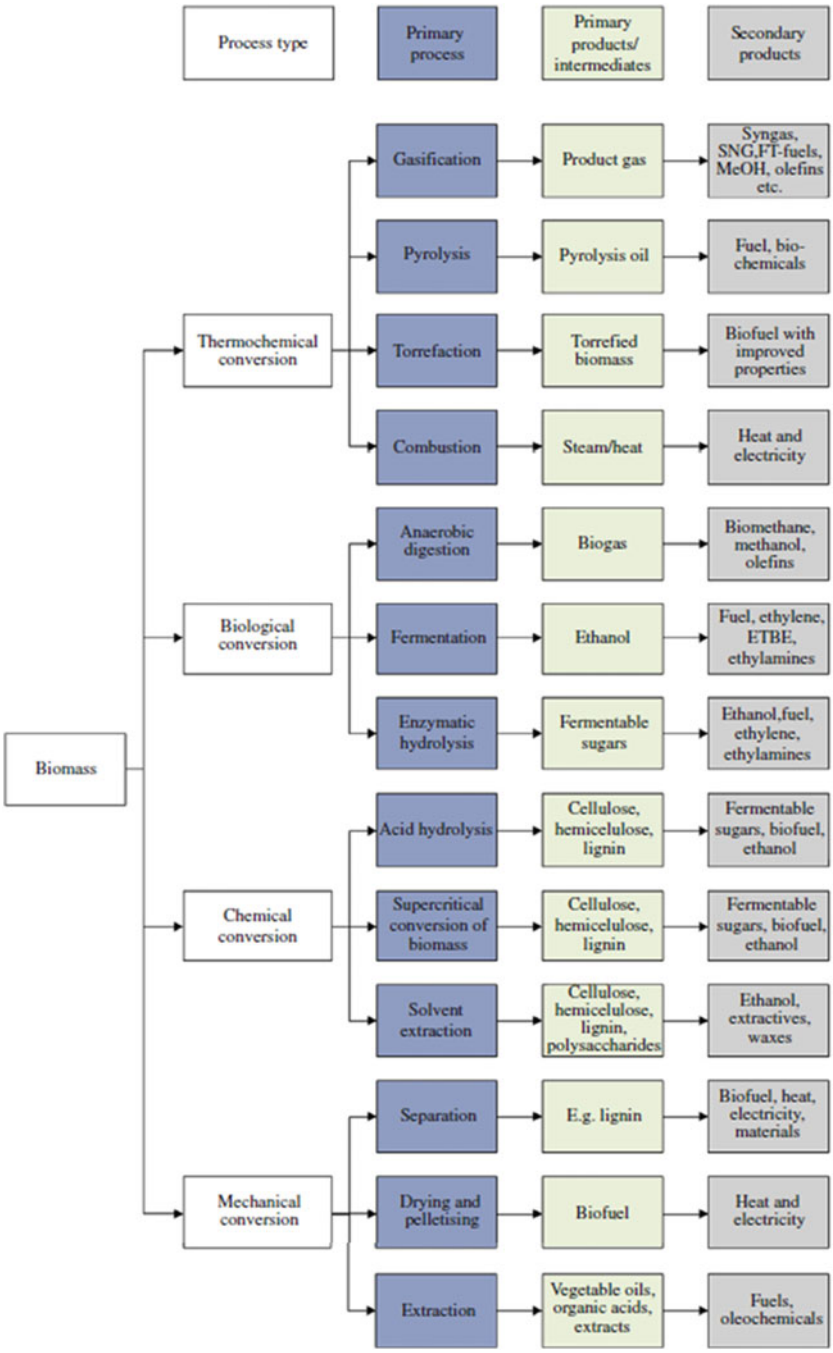

Fig. 4.3 Schematic structure of biomass conversion processes and potential products (Hackl and Harvey 2010). (Source: Reproduced with the permission of authors, Dr. Roman Hackl and Prof. Simon Harvey, Department of Energy and Environment, Division of Heat and Power Technology Chalmers University of Technology)

beta-1,4 bonds of the structural chains generating available free end (Kumar and Naraian 2019). Their major sources are different microorganisms like fungus and algae that can vary based on enzymatic actions. For example, a fungal endoglucanase contains a catalytic module but may or may not contain a non-catalytic carbohydrate-binding module, but bacterial endoglucanase may contain multiple numbers of catalytic and non-catalytic modules along with some unknown modules (Zhang and Zhang 2013).

Exoglucanases show activities on non-reducing and reducing cellulose ends and form final compound glucose. Cellobiohydrolases are one of the major exoglucanases which are produced by aerobic and anaerobic types of bacteria and fungus. Family 48 exoglucanase participates in crystalline cellulose hydrolysis. Exoglucanase mainly participates in the cellulolytic system and facilitates the production of cellobiose which is converted to glucose by beta-glucosidases (Zhang and Zhang 2013).

Beta-glucosidases facilitate the hydrolysis of glycosidic bonds to convert non-reducing residues beta-glucoside and oligosaccharide into glucose. They have particular active sites helping them to get combined with non-reducing glucose and to separate them from cellodextrins and cellobiose. It degrades cellobiose which is a common inhibitor of both exo and endoglucanases.

4.3.1.1.1.1 Cellulase Effectivity Improvement

Some methods are used to improve cellulase action to attain a good hydrolysis rate by detailed analysis of protein structure and molecular modelling. The change of amino corrosive grouping can be accomplished through site-coordinated mutagenesis, trade of components of auxiliary construction, and even trade of entire areas and additionally age of combination proteins. The confidence in the force of an objective plan depends on the conviction that our present logical information is adequate to foresee work from the structure. This mutagenesis is used to find out the enzyme's structural activity and its reaction mechanism. From experiment and computer modeling, using mutagenesis the activity of the cellulase T. Fusca, Cel9A on soluble and amorphous cellulose can be increased by 38–41% (Zhang and Zhang 2013). Although notwithstanding proceeding with the attempt to upgrade non-complexed cellulase exhibitions, there are no broad principles and use for improving cellulase action on strong cellulose substrates.

Directed evolution process is commonly used to enhance enzyme activity and this is a protein engineering tool to identify suitable mutant enzymes. Determination and screening are important. Determination is constantly liked over screening since it has a few significantly higher efficiencies than does screening. The choice is based on the connection between objective quality and its encrypting item which presents specific benefits to its maker. Screening can be classified into two classes: working with screening, which recognizes freaks based on particular aggregates, like chromosphere delivery or radiance framing, and arbitrary screening, which picks freaks haphazardly. One example of the first screening method is the evaluation of endoglucanase on rigid plates having carboxymethylcellulose (CMC) (Zhang and

Zhang 2013). Arbitrary screening is applied when in the process the first screening method is not suitable.

Endoglucanase Stability Improvement: For proper stabilization of endoglucanase, particular enzymes have to be secreted on the surface of the microorganism, e.g., for *Escherichia coli*, a combination of main protein with ice-nucleation protein is effective for the secretion of enzymes (Zhang and Zhang 2013). The half-life season of thermal inactivation of freak Cel5A (family 5 glycoside hydrolase) melded to CBM-3 is essentially drawn out during a combination of protein molecules bounce back before thermal inactivation to the insoluble substrates. The expansion of a catalytic binding module-3 on the N end or a module 2 on the C end doesn't impact the compound exercises on the dissolvable substrate CMC however definitely diminishes the exercises on the recovered indistinct, insoluble cellulose, and microcrystalline cellulose (Zhang and Zhang 2013). The finally produced Cel5A has almost the same characteristics but it extends the half time of thermal inactivation on CMC.

Glucosidase Stability Improvement: For the stability improvement, the selection of perfect mutant is done via series of determination and selection iterations to find thermally stable beta-glucosidases. At first, the selection process is done by the experiment of glucosidases on the *E. coli,* and the cells indicating actively grown glucosidases on a synthetic medium with cellobiose are collected. Similar molecules on determination specimens are copied by utilizing nylon films. After heating, the nylon films are laid on cellobiose plates with the goal that the excess exercises of thermally stable BG breaks cellobiose on the plates to glucose. *E. coli* development on the plate can use glucose to separate the thermostable beta-glucosidase (Zhang and Zhang 2013).

4.3.1.1.2 Hemicellulase

Hemicellulases are an important group of enzymes that hydrolyze hemicellulose, (Shallom and Shoham 2003) a commonly found polysaccharide, like xylanase, xyloglucans, arabinoxylans, glucomannans commonly found in biomass derived from plants (Lü 2021). Its activities are similar to cellulases due to the presence of beta-1,4 glycosidic bonds. Hemicellulases have a modular protein, catalytic module and may also have some other functional modules. Modules like carbohydrate-binding module (CBM) help in targeting the enzymes to the insoluble polysaccharides and dockerin modules that facilitate the catalytic domain parts binding through interactions named cohesion dockerin modules to surface of the microbial cell (Lü 2021). Catalytic modules like glycoside hydrolase hydrolyze glycosidic bonds.

Source of hemicellulases are microbes like saprophytic ones, also fungus, yeast, algae, protozoans, bacteria, etc. Some of the important species are filamentous fungi and bacteria like *Clostridial* and others.

4.3.1.1.2.1 Hemicellulase Effectivity Improvement

Some research work has been done for the enhancement of hemicellulase in the last few years. Most hemicellulolytic systems are controlled by the presence of carbon sources. If the basal level in extracellular enzymes decreases than its normal value, then some inducers, e.g., mono and disaccharides, or relatively bigger size oligosaccharides, e.g., aldotetraouronic acid, is added to maintain the levels. (Shallom and Shoham 2003).

A couple of hemicellulolytic regulatory protein atoms are perceived and depicted, which include XlnR, a transcriptional activator, the carbon catabolite repressor protein molecule CreA, and pH-authoritative protein molecule PacC in microorganisms like a parasite (de Vries and Visser 2001; Shallom and Shoham 2003).

In bacterial microorganisms, most of the hemicellulolytic systems are inducible (Shallom and Shoham 2003). In *G. stearothermophilus* T-6, solitary operon has 11 qualities associated with utilization of glucuronic acid (Shallom and Shoham 2003).The operon incorporates 3 qualities that arrange a carrying framework for corrosive like aldotetraouronic and the leading protein UxuR, which has a place with the GntR family. Curiously, the uxuR quality comes up short on the sanctioned GGAGG arrangement in the binding areas of the ribosome, probably to restrict the interpretation. Some enzymes have a carbohydrate-binding module (CBM) and show the activity of xylan esterase, e.g., Xyn11A, which is present in the plant's cell walls to hydrolyze the xylan backbone (Shallom and Shoham 2003).

4.3.1.1.3 Xylanase

For hydrolyzing glycosidic bonds of xylan, xylanases act as a major catalyst, e.g., beta-1,4 xylanases increase the rate of hydrolysis of xylan beta-1,4 glycosidic bonds. After this degradation process, the products produced from hydrolysis are treated with further other enzymes, e.g., beta-xylosidase. These types of enzymes basically belong to carbohydrates active enzymes. Most of them are endo-xylanase, and few of them are exo-xylanase produced by some bacterial microorganism. Both types of xylanases are mesophilic in nature. Endo-acting beta-1,4-xylanase breaks xylan into smaller xylobiose and xylooligomers. According to their properties, they fall under the glycoside hydrolase family F & G. Xylanases are classified into the glycoside hydrolase families 3, 5, 7, 8, 10, 11, 30, 39, 43, 52, and 54 (Kumar and Naraian 2019). Most of the xylanases used are from families 10 and 11 (Yeoman et al. 2010).

Bacterial-originated xylanases are used mostly in industrial biorefineries. Endo-acting xylanases produced from bacteria, fungi, and plant cells fall into the family no: 10 of glycoside hydrolase, whereas xylanases produced from bacteria and fungi are categorized in the family no: 11 (Kumar and Naraian 2019). Operational temperature for xylanases to operate 60–75° centigrade in a pH range of 6–10 where they show their maximum enzyme activity (Yeoman et al. 2010), e.g., *Bacillus halodurans* secretes xylanase enzymes which show its maximum activities

at 75-degree centigrade temperature at a pH value of 9- and 70-degree centigrade temperature at pH of 10 (Yeoman et al. 2010).

4.3.1.1.3.1 Xylanase Effectivity Improvement

Xylanase activity is controlled by activation and repression processes which are controlled by carbon sources availability (catalytic mechanism) and inducers (e.g., xylose, arabinose) for their substrates and repressor during high sugar concentration. The concentration of polysaccharides regulates the secretion of the respective hydrolyzing enzymes and controls their activity. These iso-enzymes regulate their activation (Yeoman et al. 2010; Trevizano et al. 2012).

To enhance thermal stability and activity of xylanase, a normally error-prone PCR system is used in directed evaluation which results in an increase in the stability of the enzyme from *T. lanuginosus*, a second-generation mutant but they reduce enzyme activity of the enzyme. But this problem can be solved by using proper mutants by the error-prone PCR system (Sriprang et al. 2006).

4.3.1.2 Enzyme Stability

The catalytic process goes through a collection of denaturation responses during creation and application in biorefineries. The denaturation process is the unfurling of catalyst tertiary design to a disarranged polypeptide in which key deposits are at this point not adjusted intently enough for proceeded with interest in utilitarian or construction settling communications. Due to chemical changes in protein, its activity reduces continuously and gradually becomes inactive. The molecular process results in two distinct protein stability, i.e., thermodynamic stability and kinematic stability. The first one treats the resistance of the folded and geometrically conformed to be denatured and kinetic stability treats the resistance to be inactive irreversibly.

The enzyme can be found in the region of local active state N that is in balance with the part of the denatured, enzymatically inactive U state. At high temperatures, it will, in general, unroll in a cyclic process. The softening temperature (Tm) of the protein is the temperature at which both the local active state and enzymatically will be the same. This temperature may change from low temperature to a relatively high one, yet in dilute solution, it lies in a range of 60–80 °C (Iyer and Ananthanarayan 2008).

Studies on enzyme characteristics and its activation and inactivation, type of the responses whether it is reversible or irreversible help in better understanding of the enzyme stabilization and control over its properties. One methodology is the observation of the impact of these denaturants on the movement of the target compound and the product formed. Some denaturants are mentioned in Table 4.1.

The stability of enzyme molecules is very important to maintain their efficiency. As per the Lumry-Eyring theory, inactivation of enzymes generally occurs in two probable steps—the original enzyme unrolling reversibly and then through an

Table 4.1 Different types of denaturants, their target product and the final product formed (Iyer and Ananthanarayan 2008)

Type of denaturants	Target product	Final element
Heat	Hydrogen bonds	Highly disordered structure aggregates
Mechanical forces	Solvated groups Void volume	Highly disordered structure Inactive monomers
Radiation	Functional groups like cysteine, peptide bonds	Oligopeptides, amino acids
Cold	Hydrophobic bonds, Solvated groups	Aggregates
Biological denaturation process	Peptide bonds	Oligopeptides, amino acids

irreversibly kinetic pathway due to which enzyme molecules get aggregated. The initial step is because of the function of the communications that keep up with the local design of the catalyst. The regular advancement of the primitive construction towards thermodynamically stable protein macromolecules is the next step. Factors like chemical compounds, acidic or basic nature, and high or low temperature affect the activity of an enzyme by changing its configuration. For multimeric proteins with or without dynamic totals, smaller unit separation is termed inactivation interaction. Inactivate process works in a cycle including responses like separation of smaller units of the multimeric compound, ternary or optional designs denaturation, accumulation, and substance deterioration. In general, multimeric catalysts are steadier because of their advanced inflexibility (Iyer and Ananthanarayan 2008).

4.3.1.2.1 Enzyme Stabilization Methods

Application of enzymes depends on different stability parameters like temperature, pressure, high salt concentration, and extreme pH (Table 4.2). In industry, enzymatic reactions are operated at high temperature, high salt concentration, and in the presence of alkaline medium and surfactants which are mentioned here (Klibanov 1983; Iyer and Ananthanarayan 2008; Lü 2021).

Stability due to temperature: The rate of an enzymatic reaction highly increases at a range of temperature 25–75 °C (Klibanov 1983). It also reduces bacterial contamination and lowers the viscosity of the reaction medium. Though there are some complications in the process like high cost, complex heat resistant process equipment design, and appropriate choice of enzymes, it is important to operate the process at high temperature to increase enzyme activity (Iyer and Ananthanarayan 2008).

Stability due to pressure: Pressure is one of the denaturants that stabilize enzyme activity. Its effectiveness depends on the structure and reaction process of enzymes, and the magnitude of the forces. Pressure causes the dissociation of

Table 4.2 Description and role of some important parameters associated with chemical process and bioprocesses highlighting the importance of biocatalyzed reactions

S. No	Parameter	Chemical process	Bioprocess
1	Process operating temperature	68–69 °C	0–15 °C
2	Product concentration	Necessary 70	Not applicable
3	Acrylamide concentration	30%	48–50%
4	Single-pass reaction yield	Almost 75%	95–100%
5	CO_2 production (kg of CO_2/kg of acrylamide)	1.5	0.3
6	Energy required (steam and electricity required in MJ/kg acrylamide)	1.9	0.4
7	By-products/wastes	Glycerol, washing water, solid residues	Nil
8	Cost-effectiveness	Less cost-effective	More cost-effective
9	Toxicity of the product formed	Can be toxic sporadically	Non-toxic

multimeric proteins (Klibanov 1983; Mevarech et al. 2000; Iyer and Ananthanarayan 2008).

Stability due to salt concentration: Halozymes, constituted of protease, amylases, lipase, and xylanase, have various catalytic activities depending on the relatively high concentration of salts present and have an acidic nature. The high unfavorable surface charge of its protein molecule renders solvents more adaptable at high salt fixations. Strong water dipoles remove high surface charge (Mevarech et al. 2000). That is why it is important to stabilize enzymes from thermal deactivation, alkaline pH medium, surfactants, and a variety of high to relatively low temperatures. Enzyme stabilization methods are used depending on the enzyme property, reaction mechanism, industrial applications, and other factors.

4.3.1.2.1.1 Naturally Stabilizing Enzymes

High-temperature resistant microorganisms can survive in extreme conditions like extreme temperature, high-pressure areas places. Difficulties in the separation and production of the enzymes from these microorganisms are due to their fast-growing nature. Relatively high-temperature resistant proteins gain structural developments with their mesophilic accomplices— differentiation being in the fundamental plan in sections far from the powerful site with the upkeep of dynamic site congruence. *Thermoplasma acidophilum* citrate has a genuine degree of essential homology anyway 15–25% progression character. Amino destructive plan assessments have incited a couple of general insights. Thermophilic proteins exhibit a small tendency to spread out. These stable biocatalysts have larger associations (i.e., hydrogen bonds, electrostatic attractions, hydrophobic and hydrophilic attraction, metal confining) than in less consistent impetuses and an unparalleled structure (i.e., more unbendable, larger squeezing capability, lessened spreading out entropy, spatial

strain release what's the more, unfaltering quality of a-helix). The Gibbs free energy for the stability of these enzymes is quite higher (4–20 kcal/mol higher) than the mesomeric enzymes which increases their kinetic stability (Li et al. 2005). Thermophilic enzymes have high flexibility, rigidity, and high efficiency at high temperature; therefore, it is used only in the processes where high temperature is required. Some applications of thermophilic enzymes are alpha-amylase from *B. stearothermophilus*, and alkali resistance protease *B. firmus* microorganisms (Iyer and Ananthanarayan 2008). Some holoenzymes are also thermophilic enzymes like endo1,4-xylanase, beta-xylosidase are synthesized from a halophilic organism. The strength of these enzymes may reduce because of the excess amount of salts utilized, though their use in industry is still in the experimental stage (Li et al. 2005; Iyer and Ananthanarayan 2008).

4.3.1.2.1.2 Mesophilic Enzymes

4.3.1.2.1.2.1 By Modifying Protein Structure

Protein mutation varies from the structure of enzymes to the design of protein evolutions. To stabilize the rational protein, different methods are applied like stabilizing entropy by introducing prolines or other disulfide compounds. Also, to yield high protein stability and activity, different developments are done to control the protein mutations. Due to differences in amino acid arrangements in the structure, tightly bounded geometric arrangements occur along with the increase in hydrophobic nature. By observing the protein structure and by genetic mutations, different proteins are made more extreme condition resistant and stable at extreme thermal conditions. For example, yeast triosephosphate isomerase is made more thermally resistant by N781 or N78T substitution (Iyer and Ananthanarayan 2008).

4.3.1.2.1.2.2 By Chemical Modifications

Modification of enzymes chemically is still under the experimental stage. Except for some basic amino acids, this modification process is less applied in industries. This modification can be done by basically processes:

4.3.1.2.1.2.2.1 Substituting Monofunctional Proteins

In this process, amino acids, alkylated and several other substituted protein parts are chemically treated by different processes like cross-linkage methods which can make enzymes more thermally stable but this process is still under experimental process. This process mainly focuses on elucidations of amino acid arrangements and the separation of the residual parts at the activation site of the proteins (Schmid 1979). Some users of this process are the replacement of Lis residues of enzymes by Arg which facilitates the enhancement of intramolecular salt bridges (Schmid 1979).

4.3.1.2.1.2.2.2 By Grafting to Polysaccharides

In the structure of proteins, carbohydrate residual parts are linked to the amino acid at the activation surface site. These glycoproteins enhance stability, proteolysis, heat resistance, and other properties (Schmid 1979). Covalent attachment of dextran by S-triazine to protein is a result of water solubility of glycoprotein. These processed enzyme molecules exhibit enhanced kinetic properties and high pH activity.

4.3.1.2.1.2.2.3 By Grafting to Synthetic Polymers

This process is still in the experimental stage. In this procedure, enzymes are grafted to water-soluble polymers through the addition of acid anhydrides, e.g., ethylene maleic acid anhydride (Schmid 1979). This process makes enzymes more resistant to extreme conditions and increases enzyme activity. For example, PVC conjugated alpha-D-N-acetyl hexosaminidase is very helpful in the stabilization of biocatalyst and extends enzyme longevity (Iyer and Ananthanarayan 2008). The stability of enzymes can also be controlled by additives, stabilizing the solvents in the medium, and by the addition of salts.

4.3.1.2.1.2.3 Additive Stabilized Enzymes

In industry, normally enzyme stabilization processes are mainly focused on the additive stabilized enzymes that enhance protein stability. The overall process may be costly, and the stabilizing additives may cause obstacles to the reaction system, but this process has a huge application in industries and also applies to a wide variety of enzymes in biorefineries (Iyer and Ananthanarayan 2008).

4.3.1.2.1.2.4 Solvent Stabilized Enzymes

Solvent concentration has a significant effect on enzyme stability. At high concentration, protein is denatured in solvents which leads to a reduction of stability of enzymes, but at relatively low solvent concentration, i.e., less or equal to 20%, it stabilizes. Some solvents like glycerol enhance protein stability even at high concentrations and maintain protein below zero-degree centigrade temperature, these are called cryoprotectants (Schmid 1979). Even in some plants sugar, amino acids have low-temperature resistance which helps them to survive in extremely cold regions. Cryoprotectants like dimethyl sulfoxide are used in protein stabilizers and enzyme technology. Polyalcohols increase the storage stability of enzymes by reducing bacterial growth creating high osmotic pressure (Schmid 1979).

4.3.1.2.1.2.5 Addition of Salts

Salts concentration, their polarity effect on the rate of the enzymatic reactions. For divalent cations (Ca^{+2} from $CaCl_2$), a small concentration of these salts increases the enzyme stability (Schmid 1979). As per the "Hoffmeister lyotropic series" of the

effect of salt concentration on the enzymes, cations are effective as additives, and their effect of stabilizing decreases with the anions (Iyer and Ananthanarayan 2008). Thermolysin, a relatively high-temperature resistant peptidase extracted from *B. stearothermophilus* exhibits ligands formation that is bound to asparagine and glutamine carboxyl groups. This ion increases molecular stiffness, although its application is highly specific in industry.

4.4 Optimization of Enzymatic Biochemical Process

The optimization is an integral asset that can quickly further develop titers, yields, and efficiency. The optimization is done for a few stages utilizing different strategies. Hydrolysis and fermentation measures are the most fundamental advances associated with a biorefinery. Different examinations across the globe have been finished utilizing enzymes for these biorefineries. Various optimization strategies are accessible for improvement of fermentation medium and fermentation cycle conditions like factorial design, Plackett and Burman design, borrowing, component swapping, biological mimicry, one-factor-at-a-time, response surface methodology, evolutionary operation factorial design. Every optimization strategy enjoys its benefits and burdens.

4.4.1 Enzymatic Hydrolysis

Enzymatic hydrolysis is viewed as the most encouraging strategy for the saccharification of biomass for biofuel production, where monosaccharides are produced by the hydrolysis of polysaccharides. Enzymatic hydrolysis needs a relatively lower temperature and milder functional conditions (around under the temperature of 40–50 °C and pH of 4–5) in comparison to the general followed acid hydrolysis method. A class of enzymes referred to as cellulase is utilized as a catalyst in the hydrolysis of cellulose. These compounds can be delivered by fungi, for example, *Trichoderma reesei* and *Aspergillus niger,* or by bacteria, for example, *Clostridium cellulovoran*s (Arai et al. 2006). The enzymatic hydrolysis can be affected by substrate and end product concentration, catalyst action, and reaction conditions. It is notable that the conjugation activity of cellulases and hemicellulases can build the pace of enzymatic hydrolysis and accomplish higher sugar formation. Hemicellulases work with cellulose hydrolysis by exposing the cellulosic fibers, consequently making them more open to cellulases (Shallom and Shoham 2003). Different enhancement strategies are utilized for different feedstock. Lignocellulosic biomass, for example, Wheat Straw (WS) is an economical, bountiful, broadly accessible resource. Response Surface Methodology (RSM) is used for the optimization of enzymatic hydrolysis by pretreatment of Wheat Straw (WS) using alkaline peroxide. The alkaline peroxide technique is used to delignify the WS followed by

enzymatic hydrolysis of WS. Various factors of pretreated WS hydrolysis yield are studied like response time, surfactant concentrations, substrate concentration, and enzyme loading (Qi et al. 2009). To lessen the expense of the production process and high production of fermentable sugar, dynamic simulations of fed-batch enzymatic hydrolysis from alkali-pretreated sugarcane bagasse are done (Fan et al. 1987). Saccharification with enzymes is a significant advance for the greatest sugar yield, substrate loading, pH, and temperature comprising significant boundaries for optimization of the saccharification process. Advancement of the saccharification process is exceptionally difficult as it is important to get a high return of monomeric sugars which can be changed over into bioethanol by maturation measure.

4.4.2 Fermentation

Regular biomass natural changes are fermentation and anaerobic digestion. In biomass fermentation, microorganisms are utilized to naturally change over bio feedstock into biofuels, (for example, bioethanol and biobutanol), synthetic substances, materials, or flammable gases. For example, starch and molasses are normally utilized as feedstocks to create ethanol utilizing fermentation, during which the sugars (e.g., glucose, maltose, and sucrose) are converted into bioethanol or biobutanol. The principal functional factors influencing ethanol yield and fermentation effectiveness are osmotic pressing factors and the presence and evacuation of some by-products (harmful to yeasts) of hydrolysis responses and yeast metabolism. Selecting fermentation medium and yeast type, detoxifying the hydrolysate, and isolating/eliminating the fermentation side-products are powerful measures to optimize the fermentation yield. An essential constraint in biomass fermentation is that yeasts can't straightforwardly change over cellulose, hemicellulose, and related parts intrinsic in biomass to ethanol (Johnson and Echavarri-Erasun 2011). Cellulose and hemicellulose expect digestion into fermentable sugars before they can be used by yeasts. Cellulose and hemicellulose need to be converted into fermentable sugars before they can be used by the yeasts. The lignin part in some biomass is astoundingly refractory to digestion and transformation to bioproducts by most microorganisms, with the exception of white-rot fungi; however, digestion by these fungi too is moderate and inefficient (Lynd et al. 2002; Hahn-Hägerdal et al. 2006; Jeffries 2006; Jeffries et al. 2007). Essential systems are being sought-after for fermentation of cellulosic biomass by yeast-like improvement and enhancement of xylose and other pentose used by *Schef. sstipitis* or other pentose fermenting yeasts and presentation of various pathways for fermentation of xylose and different pentoses into *S. cerevisiae* to make use of its notable strong fermentation limit and high ethanol tolerance (Ostergaard et al. 2000; Lynd et al. 2002; Saha 2003; Jeffries 2006; Van Maris et al. 2006; Chu and Lee 2007).

4.4.2.1 Bioethanol

The conversion of renewable materials into helpful higher-esteem compounds isn't new. For example, since 6000 BC sugarcane has been utilized in the creation of bioethanol from different biomaterials. The old Egyptians produced liquor by aging vegetative materials. Likewise in old occasions, the Chinese found the art of distillation, which builds the concentration of alcohol in fermented solution (Demirbas 2009). Hexoses and pentoses can be acquired from the hydrolyzates of lignocellulosic materials. Contingent upon the lignocellulosic source, the hydrolyzate ordinarily comprises xylose, arabinose, glucose, galactose, mannose, and rhamnose.

Pentosan to pentose:

$$(CH_5O_8H_4)_n + nH_2O \rightarrow nC_5H_{10}O_5 \tag{4.4}$$

Hexosan to hexose:

$$(C_6H_{10}O_5)_n + nH_2O \rightarrow nC_6H_{12}O_6 \tag{4.5}$$

The selection of microbes for ethanol fermentation is based on performance parameters and different prerequisites like similarity with existing products, cycles, and equipment. The performance parameters for fermentation are yield, temperature range, substrate usage, pH range, alcohol tolerance, osmotic resistance, explicitness, growth rate, genetic stability, and inhibitor tolerance, productivity (Demirbas 2005). The yeast *Saccharomyces cerevisiae* and the bacterium *Zymomonas mobilis* are the most popular microbes for ethanol production from hexoses (Claassen et al. 1999). *S. cerevisiae* can effectively mature basic hexoses like d-mannose, d-glucose, and d-galactose and also disaccharides like sucrose and maltose and can yield bioethanol convergences of up to 20% (v/v) in the fermentation broth (Banerjee et al. 2010). Metabolic designing and displaying of the metabolic pathways associated with the fermentation of pentoses and other sources of carbon are being used to upgrade fermentation efficiency (Ostergaard et al. 2000; Alper et al. 2006; Stephanopoulos 2007). The accessibility of the genome sequences from *Schef. stipitis* and different yeasts has given significant assets to the progress of biomass usage. Checked enhancements have been made in the fermentation of cellulosic hydrolysates by yeasts, still, the cycles have not arrived at the phase of monetary feasibility and industrialization. Ethanologenic microscopic organisms, for example, *Klebsiella oxytoca, Escherichia coli, and Zymomonas mobilis*, have recently caught an eye for industrial application. Enteric microbes, for example, *Escherichia coli* and *Klebsiella oxytoca,* are extremely alluring starting microorganisms in the native strategy for building microorganisms that ferment sugars in cellulosic biomass as they have a wide substrate go and can use both hexose and pentose sugars in cellulosic biomass hydrolysate(Lynd et al. 1999). *K. oxytoc*a has the native ability to metabolize xylobiose, cellobiose, cellotriose, and xylotriose as they can utilize all

the monomeric sugars in the lignocellulosic biomass (Wood and Ingram 1992). *Z. mobilis*, a Gram-negative bacterium, is all around perceived for its ability to produce a high return of bioethanol as a result of the anaerobic use of d-glucose utilizing the Entner–Doudoroff (ED) pathway (Girio et al. 2010). Consolidated bioprocessing (CBP) highlights the blend of cellulase production, enzymatic cellulose hydrolysis, and fermentation in a solitary process step. CBP can give minimal expense for ethanol production by using cellulosic biomaterial. To understand the capability of CBP, such microorganisms are required that can consume the cellulose at a higher rate leading to increased production of bioethanol. The improvement of CBP microorganisms through the local and recombinant methodologies has accomplished generous advancement as it is a breakthrough for low-cost biomass processing (Lynd et al. 1996).

4.4.2.2 Biobutanol

Biobutanol is an excellent fluid fuel that can be mixed with gasoline. It tends to be mixed with practically no restrictions and can be utilized without altering the motors of existing engine vehicles (Dahman et al. 2019). To compete viably with the butanol produced by the petrochemicals, the dibutanol industry must carry out the butanol fermentation under streamlined conditions. Acetone Butanol Ethanol (ABE) fermentation, likewise alluded to as "solvent fermentation," was started in the early piece of the twentieth century. The ABE fermentation comprises anaerobic microscopic organisms, which are native to the *genus clostridia* to ferment treated biomass and changes the two sugars to acetone, butanol, and ethanol. This cycle utilizing genetically changed strains of *Clostridium acetobutylicum* or *Clostridium beijerinckii* strains of the *clostridia* family is an effective process of biochemical conversion of biomass to biofuel. The focal point of this field of exploration is to create butanol rather than ethanol on account of its benefits over bioethanol.

Fermentation of mixed alcohols from sugars by Clostridium can be represented by

$$7CH_2O \rightarrow C_4H_9OH(\text{butanol}) + 3CO_2 + 2H_2 \tag{4.6}$$

$$5CH_2O \rightarrow C_3H_7OH(\text{propanol}) + 2CO_2 + H_2 \tag{4.7}$$

$$3CH_2O \rightarrow C_2H_5OH(\text{ethanol}) + CO_2 \tag{4.8}$$

$$5CH_2O \rightarrow CH_3COCH_3(\text{acetone}) + 2CO_2 + 2H_2 \tag{4.9}$$

Butanol has a lower vapor pressure and lower water dissolvability as well as higher energy content than bioethanol. It can likewise be utilized at a higher rate in current motors without any changes. Recent progress has been made in butanol fermentation, including upstream and downstream processing. Using excess carbon in limited nitrogen is needed to accomplish a significant level of solvent production (Madihah et al. 2001). Classical fed-batch and continuous cultivation don't appear to

be financially practical as a result of solvent toxicity and the biphasic nature of acetone butanol fermentation, separately. Fed-batch cultures are combined with an in situ recovery process to solve the issue (Ezeji et al. 2004). Butanol has been created from different natural materials including corn, potato, and molasses. The current classification may give a more cost-competitive and environment-friendly biobutanol production process. It is delivered from corn or sugarcane, lignocellulosic-and syngas-based butanol has been broadly studied. A significant factor affecting the expense of biobutanol production is fermentation substrate. The various secrete enzymes that work with the breakdown of polymeric carbohydrates into monomers, which make these great substrates for biobutanol production are secreted by *Clostridia* (Ezeji et al. 2007). The significant expense of traditional starch-or sugar-based substrates like maize, wheat, millet, rye, and molasses is the main monetary factor, which influences the production and use of the ABE fermentation. A variety of sustainable substrates with minimal expense, for example, lignocellulose, cellulose, and hemicellulose have a splendid future and will be effectively applied to produce ABE fermentation. Optimization techniques which inspect the fermentation factors as individual components are tedious. Then again, the conceivable collaboration of fermentation factors is normally overlooked. Among the statistical experimental designs that have been used in looking for ideal conditions for a multivariable system, a few analysts report that Response Surface Methodology (RSM) can be effectively applied to improve alcoholic fermentation. Normally this strategy is utilized for the optimization of culture conditions and the determination of ideal qualities for processing parameters, like pH, temperature, air circulation, and feeding rate, among others (Zertuche and Zall 1985). The interaction between glucose concentration, butyric corrosive addition, and C/N proportion is researched to advance butanol production utilizing RSM. Contingent upon substrate cost, the fermentation process can be optimized for either yield or productivity. Since more significant levels of starch are needed for increased solvent productivity, it will be alluring to augment such productivity just when an attainably valued substrate is available. More prominent solvent concentrations in a batch system will prompt decreased refining costs. Butanol has likewise been delivered from minimal expense waste products, for example, waste from packing peanuts and agricultural waste in the form of starch. Lesser grade glycerol is being additionally utilized to produce butanol in a chemostat culture of *C. acetobutylicum*, which brings higher return and productivity (Andrade and Vasconcelos 2003). In the optimization of complex industrial processes, there exist various potentially ideal solutions which are best acquired using Multi-Objective Optimization (MOO).

4.5 Critical Evaluation of Enzyme Activity in Biorefinery

4.5.1 Merits and Demerits of Enzyme Technology in Integrated Biorefinery

4.5.1.1 Merits of Enzyme Technology

Enzyme technology has assumed a significant part in numerous biorefineries. Enzymes are likewise significant in decreasing both energy utilization and ecological contamination. The utilization of enzyme technology much of the time brings about many advantages that can't be acquired with conventional compound treatment. These frequently incorporate higher item quality and lower fabricating costs, and less waste and diminished energy utilization. More conventional substance medicines are by and large vague, not in every case effortlessly controlled, and may make cruel conditions. Regularly they produce unwanted side outcomes and additionally garbage removal issues. How much an ideal specialized impact is accomplished by a compound can be controlled through different means, like portion, temperature, and time. Since enzymes are biocatalysts, the sum added to achieve a response is somewhat little, e.g., enzymes used for the food industry normally are less than or equal to 0.1%, but most of them normally cannot be recovered at the end of the processes. Enzyme technology is specific to a particular reaction needed. The amount of residual products is reduced, and the wastage can be controlled.

This property of enzymes reduces the production cost of the products. Enzyme technology can frequently supplant synthetic compounds or cycles that current security or ecological issues like it can reduce the use of sulfur compounds, acid, alkalis, i.e., oxidizing agents in the starch producing industry. Catalysts likewise add to more secure operations in the industry through the disposal of chemical compounds along with the productions. These advantages make the use of enzymes more profitable for the industry (ETI 2001).

4.5.1.2 Demerits of Enzyme Technology

Enzymes need to operate in an optimum environment. Some enzymes require an optimum temperature of 37 °C just like amylase enzymes that break down starch; however, some enzymes require a relatively high temperature to operate such as certain enzymes in some bacteria. If there is a sudden change in the reaction medium or process, then the enzyme activity will be reduced and it will be denatured. To resist this inactivity, the temperature should be maintained up to a certain limit or enzymes can be made thermally stable chemically, but these processes sometimes become costly. On the off chance that the reactant that is being used with the biocatalyst has an unexpected pH in comparison to the compound that is found in the enzyme, the dynamic site (the site where particles tie on a protein for a response to occur) would be adjusted and the catalyst would at this point don't be proficient or

denatured. Another burden that is generally talked about is the manner by which water is required in sufficient sum for biocatalysts measure. Water goes about as dissolvable in light of the fact that it has a high edge of boiling over and that of the warmth of vaporization which would assist the protein with working ideally. This huge amount of water supply and heat treatment may increase the production cost and complexity of the reactor design. Unwashed enzymes can cause allergic infections sometimes, but this can be controlled by a proper enzyme recovery system. Enzymes used in processes can pollute other substances which can reduce the reaction rate. But if these disadvantages are properly controlled in the process, enzyme technology is the most efficient method in the biorefineries.

4.6 Conclusion

The biorefinery offers a lot of freedom to create a variety of products and organic synthetics from natural biomass. The biorefinery can be visualized through different biomass conversion processes. Mechanical processes, that basically deal with the size reduction of the biomass including chipping, grinding, milling and refining operations, chemical processes, including Fisher-Tropsch synthesis, hydrolysis and transesterification, and methanization. Biochemical processes dealing with the production of various biofuels from the natural biomass. And the thermochemical processes incorporate ignition/burning, liquefaction, gasification, and pyrolysis. This process involves the conversion of lignocellulosic biomass through pyrolysis and gasification. All these processes give a variety of numerous primary and secondary products such as biogas, syngas, cellulose, hemicellulose, lignin, biofuels, etc., and simultaneously produce heat and electricity.

The effectiveness of all these processes can be increased by the introduction of enzymes in the biorefinery. Cellulases and hemicellulases produced from the various microbes such as fungi, bacteria, protozoa, and yeasts are widely used in biomass processing by saccharification. Hemicellulases also hydrolyze hemicellulose. Increasing the effectiveness of these enzymes can increase the hydrolysis rate. By direct evaluation and analyzing the detailed structure of the protein and by experiment and computer modeling, the effectivity of the cellulases can be increased. Along with that, some research works are being done to increase the effectiveness of hemicellulases also. To maintain the process efficacy, it becomes very important to maintain the stability of enzymes and prevent denaturation which occurs due to unrolling of enzymatic structure. The enzyme stability can be maintained by maintaining optimum temperature conditions (25–75 °C), optimum pressure, and salt concentration. The enzyme stabilization method also depends upon the property of the enzymes and reaction mechanisms.

With the introduction of enzymes in biorefineries, we got a plethora of different products. But along with the use, it becomes very necessary to go for more optimized enzymatic processes. Bio-catalytic hydrolysis is a well-accepted method for biomass saccharification over acid hydrolysis. The optimization of enzymatic hydrolysis is

done via response surface methodology. Using response surface methodology (RSM), optimized butanol production can be achieved. To optimize complex industrial processes, there are various potentially optimal solutions obtained using multi-objective optimization.

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

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Chapter 5
Process Efficacy in Cassava-Based Biorefinery: Scalable Process Technology for the Development of Green Monomer D-Lactic Acid

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Abstract Process integration approaches in biorefinery are crucial for sustainable development. Research related to the sustainable production of biodegradable plastics especially polylactic acid is gaining momentum worldwide. The stereospecific nature and economic viability of the lactic acid monomer are crucial for poly (lactic acid). These surge the concepts of "green monomers" from biorefineries especially D-Lactic acid (DLA). Globally, the utilization of sustainable feedstock has been preferred as a cost-effective and eco-efficient strategy for value-added product synthesis. The underutilized agri-food industries are facing economical threats from their stakeholders, especially cassava-based industries. The development of an effective techno-economic strategy through enzyme-based greener process integration approaches will boost the economy. The enantiomeric purity of DLA is a crucial factor for its industrial applications, and the greatest demand exists for the optically pure isomers which can be achieved only by scalable enzymatic technology. DLA production could be a potential strategy for value addition for the generated cassava fibrous waste (CFW) at different stages. The objective of this case study is to address the scalability aspects of the uncherished resource potential

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of CFW towards enhanced DLA production by imparting enzymatic process intensification at different stages of integrated cassava biorefinery, especially in terms of hydrolysis, fermentation, and purification.

Keywords Biorefinery · D-Lactic acid · Enzymatic process integration · Cassava

Abbreviations

CFW	Cassava fibrous waste
DLA	D-Lactic acid
HFLAB	Homofermentative lactic acid bacteria
PDLLA	Poly DL-Lactic acid
PLA	Polylactic acid

5.1 Green Monomers—Process Integration

Access to cheap and abundant resources is a linchpin of modern industry and civilization. The diminishing fossil resources, the drastic requirement of energy demand, and alarming global warming issues favor for the development of sustainable technologies. The polymer which is a part of human life nowadays is not an exception for the green economy. In the context of this new greenish touch, highly versatile and cost-effective polymers play an essential role in sustainability. This surges the concepts of "green monomers" from biorefineries (Zhang et al. 2016b).

5.2 Cassava Biorefinery Current Scenario

Globally, the utilization of sustainable feedstocks has been preferred as a cost-effective and eco-efficient strategy for value-added product synthesis (Babu et al. 2013). The development of an effective techno-economic strategy for the conversion of sustainable feedstock to fermentable sugars plays a key role in determining the product cost and capability to produce a variety of chemicals and fuels. Cassava *(Manihot esculenta)* is the most widely cultivated root crop in the tropics and is the third-largest source of food carbohydrates in the tropics, after rice and maize which are grown across a broad range of agro-climatic conditions (Forster-Carneiro et al. 2013; Nisamaneenate et al. 2015; Sriroth et al. 2000). Cassava is a perennial tuber crop cultivated worldwide with a production of over 250 million tonnes as per FAO statistics (Fig. 5.1) (Otekunrin and Sawicka 2019). The different stages of cassava processing involve peeling, grating, fermenting, de-watering, frying, and drying which generate 25–45% of waste and are generally disposed of by the land

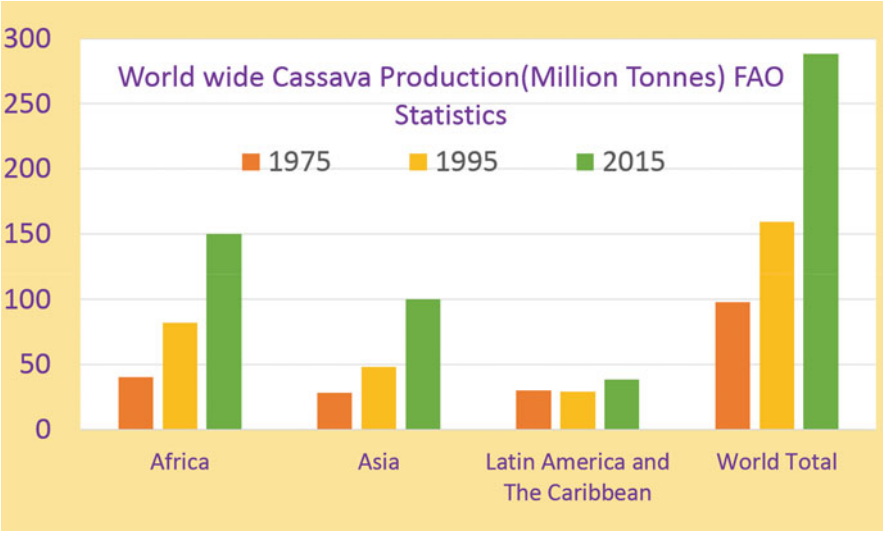

Fig. 5.1 Worldwide Cassava Production statistics

Fig. 5.2 The different stages of cassava fibrous waste generation in the sago industry

composting method (Fig. 5.2). However, composting cassava fibrous waste (CFW) poses an environmental threat leading to groundwater contamination. Valorization of CFW into value-added products would be a viable option.

Valorization of CFW into value-added products would be a viable option. Cassava fibrous waste is a solid waste generated during the processing of cassava tubers in sago industries in India which contains more than 50% weight of starch and about 600–650 tons of CFW are generated in India from the sago industry (Pandey and Soccol 2000). CFW disposed of cassava processing industries may cause a serious threat to the environment because of its high organic content.

As CFW is rich in organic content, it can be used as cheap raw material for the production of various biochemicals. CFW is a promising raw feedstock when compared to lignocellulosic biomass because of low levels of lignin and pectin and requires minimal pretreatment. CFW has been used as a raw material for the production of various value-added bioproducts, viz. glutamic acid, ethanol, pullulan, L-lactic acid. The first study of DLA by CFW was reported by Cingadi et al. (2015).

While choosing a feedstock, one needs to consider the availability, conversion capability, economics of the stock, competition, and greenhouse gas savings. Considering DLA production using CFW as a feedstock not only reduces the waste generated from the cassava starch industry but also lowers the cost of DLA production, which is one of the major components in thermostable biopolymer polylactic acid (PLA).

5.2.1 Cassava Production

Tapioca which we also known as "cassava" was once considered to be the "poor man's crop" has now upgraded to be one of the most vital crops cultivated globally in tropical and sub-tropical regions with minimal requirements. There has been an increased demand for the production of cassava in yield per hectare mostly because of its different roles in the new and the growing industries worldwide (Moshi et al. 2014). This crop does not only serve as some healthy staple food rich in starch, but it also provides food stability at the same time. This crop can be easily grown in marginal lands with well-drained soil and more moisture content. Generally, this crop requires sunlight exposure for long periods for better starch production and can be effortlessly grown with less fertilizer. A lot of factors during the cultivation process determine the starch quantity which is obtained from cassava. The production of cassava is also highly dependent on the supply of quality stem cuttings which aids in the cultivation of a greater number of cassava crops. Different varieties of cassava are being produced nowadays with improved properties like enhanced starch content, less cyanide production, drought resistant, and high yield (Nisamaneenate et al. 2015).

In the 1960s, Brazil and Africa were the major cassava producers worldwide. From the early 1990s, Africa continued supplying 50 percent of cassava produced worldwide along with Nigeria. From 2011 to 2015, the top ten countries which produced cassava were Nigeria, Thailand, Indonesia, Brazil, Ghana, and Democratic Republic of Congo, Angola, Vietnam, Cambodia, and Mozambique. Today Africa is still the leading producer of cassava as it is dependent on it for multiple purposes mostly as an emergency crop during drought and famine conditions while in Asia and Latin America it serves majorly as an industrial crop. Today there are more than 100 countries that produce cassava globally with a total output of 270 million tons and satisfying the caloric demands with the increase in the population. The total farming area for cassava is more than 18 million hectares in Africa, Latin America, and Asia.

Major cassava growing countries in Asia are India, Indonesia, Thailand, and Vietnam. India has always been known for its excellent agricultural practices starting right back from the Indus Valley Civilization. India is known for growing several staple food crops and has now attained the title of the top ten major Cassava producing countries worldwide. Cassava production in India was initiated by the Portuguese in the seventeenth century as a food crop which has now upgraded to be

Table 5.1 State-wise area and production of tapioca in India

State	Area ('000 hectares)	Production ('000 metric tonnes)
Tamil Nadu	109.56	4205.82
Kerala	72.47	2637.20
Andhra Pradesh	16.45	329.02
Nagaland	6.00	50.00
Meghalaya	5.60	30.05
Assam	4.48	38.31
Karnataka	1.00	12.90
Total (Including others)	**216.66**	**7319.13**

one of the major industrial crops grown in about 13 states by the end of the twentieth century. Kerala and Tamil Nadu are the largest producers of cassava along with emerging importance in Andhra Pradesh. It is also consumed as an important carbohydrate source in Assam, Meghalaya, and Nagaland. The data is represented in the form of Table 5.1 below. The productivity, yield, and production per unit area gradually increased from 1961 to 2000. In the year between 2012 and 2013, the total area for tapioca cultivation in India was around 216.66 thousand hectares, and the production accomplished was about 7319.13 thousand metric tons (Sugumaran et al. 2014). India is also known to export cassava processed food to countries like the United Arab Emirates, Saudi Arabia, Oman, European nations, Kuwait, and the United States of America.

The extraction of the starch granules from cassava is carried out in a sequential process in the large-scale starch and sago manufacturing industries (Saladini et al. 2016). The cell wall is ruptured by a process called rasping, using well-designed equipment containing blades that crush and grind the peeled tuber crop for the separation of the starch from the root cells. This process results in the accumulation of a residual mass. Although the rasping process is implemented more than once, still the entire starch content is not extracted completely. Cassava fibrous waste (CFW) generated during this rasping process is rich in starch and organic content and can be easily fermented to reducible sugars in presence of appropriate strains of microorganisms mostly *Lactobacillus* to produce D-lactic acid. Such a strategy for the production of DLA from the huge amount of cassava fibrous waste generated in industries will not only be feasible but also be a techno-economically superior business. CFW after enzymatic hydrolysis generates huge amounts of glucose as a carbon source which can be utilized entirely for DLA formation at a comparatively low cost (Cingadi et al. 2015).

5.2.2 *Cassava Utilization*

Cassava being one of the staple foods for many indigenous people around the globe is more prioritized than other crops like wheat, maize, rice grain, etc., which can be

related to the fact that growing cassava is more advantageous and easier than other crops which need to fulfill certain requirements for cultivation (Tumwesigye et al. 2016). The demands for the production of cassava have increased duly with the expansion in population and urbanization. Human consumption being the major usage of cassava in the poor section of our community, its huge variety of derivatives is being marketed in various food and non-food industries. More than 60% of the cassava produced worldwide is consumed as flour and fermented food. Starch granules extracted from the tubers are refined into a fine powder and used as a raw material in the production of biofuels, biopolymers, adhesives, tanning of leather, animal feed, paper, etc., and as edible items like sago, garri, chips, flour, etc. (Coker and Achi 2015). Bakeries and other food and beverage industries are one of the major customers of raw starch from sago industries for the production of jellies, candies, bread, chewing gums, syrups, etc. Chemical industries utilize raw starch obtained from cassava for the manufacturing of glucose, sucrose, lactose, dextrose, fructose, cellulose, hemicellulose, and dextrin (Elemike et al. 2015). Cassava ethanol obtained from cassava serves as an excellent raw material in synthetic chemical industries and pharmaceutical industries. Cassava also has beneficial roles on human hair, skin, and health for which it has paramount usage in cosmetic industries as well.

Not only the root but cassava leaves are also consumed by many people due to their nutritious content and have applications as cattle feed. Cassava can be segregated as sweet or bitter based on its cyanohydrin content. The sweet cassava is mostly consumed directly without any prior treatment and the bitter cassava is served for various industrial purposes. Human consumption of cassava is mostly trending in developing countries than the developed countries. Statistical reports have shown that cassava consumption has increased from 76 million tons to 96 million tons from 1984 to 1994. Surveys conducted have shown that the largest markets for cassava feed products are in Netherlands, Belgium, Germany, Spain, and Portugal. Cassava being the fourth major source of starch is also leading to the expansion of the starch-based cassava market. Starch industries located in Asia are more functional than those located in Latin America and Africa (Nisamaneenate et al. 2015).

Cassava-based fermented foods like Gari, Kapok pogari, Lafun, Chick-range, etc., and also cassava bread are most prevalent in regions of West Africa. Gaplek which is dry cassava is consumed in Indonesia. Cassava in form of rice is consumed in Philippines. Macaroni (cassava noodles), tapioca pearls, and sago are consumed mostly in Asia. Beverages like Mingao and Tapioca tea are consumed in South America and Asia, respectively. Fermented cassava-based alcohol familiar as cassava beer is produced in regions of America and Africa.

5.2.3 Economic Viability

Although the cassava crop takes around 9–11 months to grow, the overall production is a cost-effective affair. The starch and the sago industries produce huge amounts of solid and effluent waste concurrently which has to be discarded appropriately in the

environment (Abd-Aziz 2002; Elemike et al. 2015; Sugumaran et al. 2014; Wei et al. 2015). As mentioned previously, disposing of such waste not only causes environmental pollution but also contaminates the groundwater table. Therefore, to make the manufacturing processes more economically viable, waste matter is converted into value-added products. Industries using cassava crops as a raw material for the production of starch can easily acquire additional benefits by implementing the manufacturing of other essential commodities. Pretreatment of the substrate used for fermentation is also an imperative contributor to the overall production cost for DLA which is not counted here for the reason that there is very less concentrations of lignocellulose biomass present in CFW. Hence, production of DLA using CFW as feedstock will be a much more economically viable approach industrially by using low-cost equipment and optimizing the process parameters for large-scale production. In addition to that, sago industries in India serve as one of the major employment opportunities for manual workers in rural areas, and the cost of the final products from such industries (sago and starch mostly) are extremely modest contrasting to the market price of DLA (Wei et al. 2015; Isikgor and Remzi Becer 2015). In general, cassava derivatives are marketed at a rate much higher than the initial investment which is made for the processing of cassava as a raw material. The development of advanced tools and machinery can also make the post-harvesting processes much easier and more cost-effective with respect to time and labor.

Commercial development of sago industries is being done at a very low rate. Sago industries in India are widely distributed in several districts in Tamil Nadu, namely Salem, Namakkal, Dharmapuri, Erode, Tiruchirappalli, Perambalur, and Thiruvannamalai districts (Pattiya 2011; Virunanon et al. 2013). A lot of development is still needed in this industry related to marketing, financial, and laboring limitations associated with it which needs to be solved along with implications of government policies. Waste generated from this industry can be beneficially utilized for the production of DLA, contributing to the enhancement of the overall profit.

5.2.4 Value Addition

Cassava-derived products promote value addition. Sago industries that were considered trivial previously have now become a salient business in the modern day. These industries not only produce sago but also produce starch which is utilized as a raw material by many other industries like textile industries, paper industries, etc., and even laundries in the household (Coker and Achi 2015; Tumwesigye et al. 2016). Linking between the rural and urban areas will ensure better marketing of the products. The overall production cost of the cassava crop is very insignificant as compared to the processing and marketing segment and the amount of waste generated from such processing units are massive which can be converted to other by-products. With the advance in research and technology, this waste generated can be converted into other marketable and essential products like biogas, ethanol, fertilizers, surfactants, adhesives, etc. (Pattiya 2011; Cheng et al. 2015). Most of

the sago industries dry the residual waste for utilization as animal feed. Several organic compounds can also be produced from industrial cassava wastes under mesophilic conditions including citric acid, lactic acid, succinic acid, VFAs, and biosurfactants (Tumwesigye et al. 2016; Elemike et al. 2015). Biofuel production using cassava pulp has also been achieved through hydrothermal treatment. Biogas production can also be carried out from different waste from cassava processing industries and can also serve as a potential energy supplier to biorefineries. Starch-based biodegradable plastics are being made for maintaining an eco-friendly environment and also for convenient usage. DLA production using CFW being a viable process is not in trend much and needs to come into the limelight (Daful and Goergens 2017). The manual labor wage designated in such industries is not exorbitant, and the value addition of cassava promotes an increment in the source of income for the farmers growing them. Exportation of cassava-based products is being conducted at high levels in foreign countries which also provide the wholesaler's remunerative rates. Research and development have even established processes where the residual sago biomass can be even utilized for the production of electricity. Hence, agro-industries establishing collaborations with research institutes will lead to the development of efficient processing units and high profit with greater value addition. Literature reports reveal that improvement in modern processing and marketing facilities will surely enhance the efficiency of such business units along with quality standardization. Proper communication, transport facilities, and storage facilities along with good planning of infrastructure will ensure more favorable outcomes.

5.3 Biomass Waste Valorization

A biorefinery is a facility that integrates biomass conversion processes and equipment to produce a variety of products like fuels, value-added chemicals, heat, and power from biomass (Mohan et al. 2016). It resembles the petroleum refinery concept where multiple fuels and products are processed from petroleum. Abundant renewable feedstocks which are carbon neutral represent biomass for the production of a variety of products (Fig. 5.3). Present waste valorization is carried under the umbrella of biorefineries using first-generation feedstocks such as corn, soybeans, and sugarcane for bioethanol and biodiesel production (Soetaert 2013). The use of second-generation feedstock such as lignocellulosic biomass, including forestry and agricultural residues, modern cell engineering, and fast conversion technologies in biorefineries favor sustainable economic growth with minimal or no negative impact on the environment.

5.3.1 Need

There are specific challenges that need to be addressed in today's biorefinery industry. Particularly, the recalcitrant nature of the lignocellulosic feedstock,

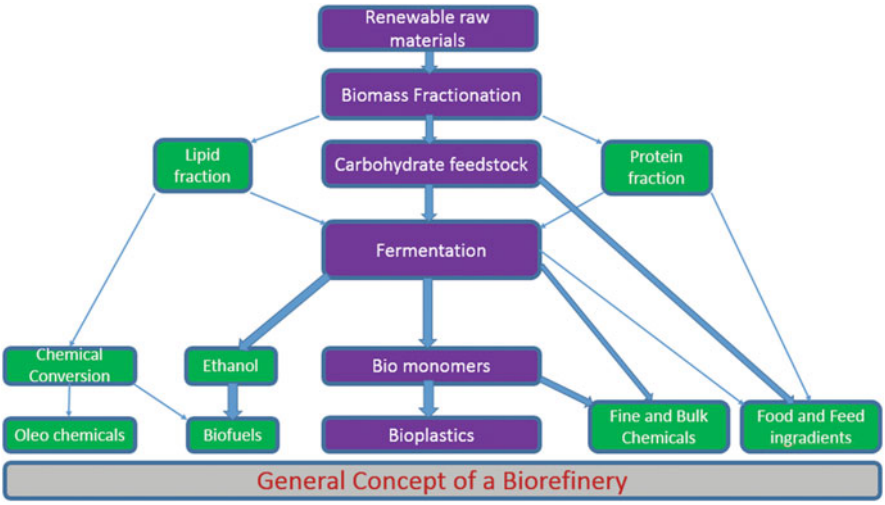

Fig. 5.3 The concept of biomass valorization to bioplastic through biorefinery

processability of pretreating and enzymatic hydrolysis, and difficulty in scaling up make the biorefining uneconomical in terms of cost and time. To improve process economics, the biorefinery should utilize all components of biomass feedstock, minimize waste generation to maximize productivity which requires the integration of technologies from various areas, including new energy crops with higher biomass yields and better processability, better and cheaper enzymes for hydrolysis, novel and improved cells and catalysts for biomass conversion to fuels, chemicals, and other marketable products, and more efficient processes for the production of these bio-based products at a commercial scale. This concludes that the selected lignocellulosic biomass involves the enzymatic treatment, thereby processing the fermentable sugars and purifying the product toward the production of optically pure DLA**.**

5.3.2 Marketability and Competitiveness

According to recently published reports in 2020, the starch industry market is projected to grow by 47.3 million metric tons, driven by 5.3% compounded growth and liquid starch with 5.4% compounded growth. The starch extracted from different sources has different properties which are inherited during value addition and further obtained in the overall property of the final product marketed. This is mainly due to the variation in amylose content and branch chain length. Cassava starch is known for its certain exceptional properties like high paste viscosity and clarity. One of the main reasons it is mostly preferred as an agro-industrial food is due to its high freeze-thaw stability. The marketability of cassava-based products will be highly dependent

on the remarkable features and quality of the final value-added product and how better it competes with other products made from other starch-containing raw materials (maize, sweet potato, rice grain, etc.). Marketability can be enhanced by monitoring the performance of the products and their impacts on society. Similarly, waste produced from cassava processing industries can also be converted into several value-added products having high market demand. The marketability of DLA is dependent totally on its level of purity, cost, and demand. Biological means for the production of DLA ensure high optical purity, and implementing CFW reduces overall production cost which will definitely enhance marketability (Solaiman et al. 2006; Daful and Goergens 2017).

Cassava starch also has certain limitations in its functionality which hinders its application industrially and can be also removed easily through better enzymatic treatments based on research and novel innovative strategies. The value addition also keeps on increasing the market value of the products by making them more appealing to the customers. The industries should decide upon a marketing team that will not only promote the cassava-based products but also create value chains. Although there are very less biorefineries actively functioning to date one of the important limitations seen in industries is that none of the biorefineries is willing to set up new setups and processes from scratch using newly made technologies and mostly consider it uneconomical. So mostly such newly made technology-based inventions using different raw materials remain as patents and fail to get commercialized. Therefore, it is preferable to work on existing technological processes and upgrade them as much as possible for better profitability and efficiency (Chang et al. 1999; Anuradha et al. 1999).

Biomass valorization concepts are known to convert biomass into value-added marketable products. Cassava-based bioproduction does not only utilize the edible tuber roots of the crop but also makes use of the leaves, stems, and fibrous residual wastes for conversion to essential products including DLA. Such a plan of action results in more profitable business outcomes. Bio-based products manufactured through biorefineries have the advantage of being more economic and eco-friendlier. With more exploration in these research fields, the production cost which is one of the barriers to DLA production can be reduced many folds. Market reports have predicted that the production of high volume low value-added products (e.g., DLA, succinic acid) can also be a beneficial marketing strategy and reduces the selling price of primary products (Börgardts et al. 1998; Sriroth et al. 2000; Xiaodong et al. 1997). The market for optically pure DLA is expanding with time for its use in the production of PLA. Most of the industries are still dependent on nonrenewable resources for the chemical synthesis of DLA marketing at a high price. On the contrary, biological means of DLA production using the fermentation method will be a better option for keeping an eye on the rising global demand. Although biorefineries are capable of marketing more than one product, producing solely DLA from CFW will also be a feasible and profitable strategy (Anuradha et al. 1999; Benthin and Villadsen 1995; Börgardts et al. 1998; Chang et al. 1999; Daful and Goergens 2017; Pinelli et al. 1997; Gonzalez et al. 2007; Södergård and Stolt 2002; von Frieling and Schügerl 1999; Xiaodong et al. 1997).

Marketability is also dependent on the consistent supply of cassava crops as raw material which has to be regulated by communicating and cooperating with the producers in the field. An abundant supply of CFW is necessary for the continuous production of DLA in biorefineries. Potential progress in the agricultural sector can also put a significant impact on the marketability and competitiveness of cassava-based starch and other products based on the annual productivity which is also having a direct impact on cassava-based industries and their waste management. In general, to achieve economies of scale, it is prioritized to have good coordination between the production team and the marketing team. Through better revolution, the cassava-based products can be diversified and well-promoted amongst all types of customers regionally as well as globally. Lastly, government policies also need to be improved for giving more relevance to promoting the cultivation of cassava as a potential industrial crop. Marketing and trade development programs can improve the partnerships between farmers and private sectors and can prove to be beneficial if implied (Wee et al. 2006).

Initiatives are being taken in India for developing the cassava market through the efforts of R & D in the private sector. Cassava-based processed foods are being manufactured and even exported to different regions. Waste generated from these plants needs to have proper waste disposal schemes, and the best way to get rid of them is by reusing them for other purposes. India has gained a high yield production of cassava with time owing to the extensive research which has been conducted. Indian local markets have huge sales of cassava processed foods like sago (sabudana), starch, chips, flour, wafer, and papad. Private companies even market machinery for processing cassava like peelers, graters, fryers, roasters, etc. But most of these industries are small-scale units and still follow the age-old traditional methods and lack the implementation of advanced technological equipment. The development of more cassava-based industries also generates relatively more cassava processed waste including CFW which needs to be disposed of properly. The utilization of abundant CFW as feedstock could satisfy the increasing demand for DLA production in a cheaper model.

5.4 Socio Environment Impact and Acceptance

Agriculture and nutrition are equally important for the socio-economic growth of developing countries. There are a lot of superstitious beliefs associated with the usage of cassava which have been solved and explained by researchers. Cassava although a nutritious edible food also has a lot of toxicity in it which needs pretreatment before consumption. The local people including the farmers producing this crop mostly consume cassava directly as the cassava leaves are a good source of protein while the roots are a good source of fiber and benefit in improved digestion. Apart from that, cassava is highly rich in essential amino acids, vitamins, and minerals and is also considered to be a good cholesterol-lowering and anti-diabetic agent. Thus, cassava does not only serve as an easily accessible, low-cost food crop

for the lower-class people but is also a good source of income for them. Similarly, with the emerging bio-economy, the local farmers are getting exposed to more market opportunities. Cassava-based biorefineries will be able to process and supply more than one value-added product from biomass which itself will be a great form of financial gain. These biorefineries will increase the number of employment opportunities especially for low-class people and in rural areas which in turn can bring improvement in the quality of life for these farmers. Biorefineries will also pave the avenue for more job opportunities for a large community of the region to be considered for installation and operation.

Biomass waste-based biorefineries have their main objectives to reduce environmental deterioration through pollution and improve the water and soil quality and also reduce the emission of greenhouse gases in the earth's atmosphere. Biorefineries also have a direct impact on the agricultural sector for the improved breeding and production of cassava crops since a stable supply of raw materials will be needed. For the establishment of a biorefinery, one major part is to get good investors for the construction of the whole business and also provide a convincing project plan. The net profit generated and the tax revenue matters are also investigated for assessing the overall economic impact. Cassava-based biorefineries are also capable of manufacturing biofuels and biogas which will serve as national energy security for countries relying totally on fossil fuels and will prove to be a good master plan for regions generating more amounts of biomass. Considering all such environmental issues along with an increase in demand for energy supply with an increased population, alternate options need to be considered and biorefineries should be accepted.

5.5 D-Lactic Acid

Polylactic acid (PLA) is one of the biodegradable polymers obtained from renewable resources. It is a thermoplastic aliphatic polyester produced by mostly ring-opening polymerization of lactide, which is a cyclic diester of lactic acid or the direct condensation of lactic acid monomers (Fig. 5.4). In 2010, PLA had the second highest consumption volume of any bioplastic in the world. The lactic acid is having two optical isomeric forms known as "Dextro (D)" rotatory and "Levo (L)" rotatory. Enantiomeric purity is important for industrial uses, and the greatest demand is for pure isomers. A deliberate blending of the enantiomers provides an effective method to control both the physical properties of polylactic acid and the rate of biodegradation. The subtle difference in stereochemistry has a drastic impact on mechanical properties and degradation which favors the production of lactic acid by biological processes rather than chemical processes which yield racemic mixtures. The production of lactic acid consists of two stages. The first one is the pretreatment where characterization and enzymatic treatment of the selected starchy-rich feedstock are carried out for the production of fermentable sugars. The second one is fermentation where the treated enzymatic hydrolysate is used in the medium formulation for fermentative production of lactic acid by the selected strain.

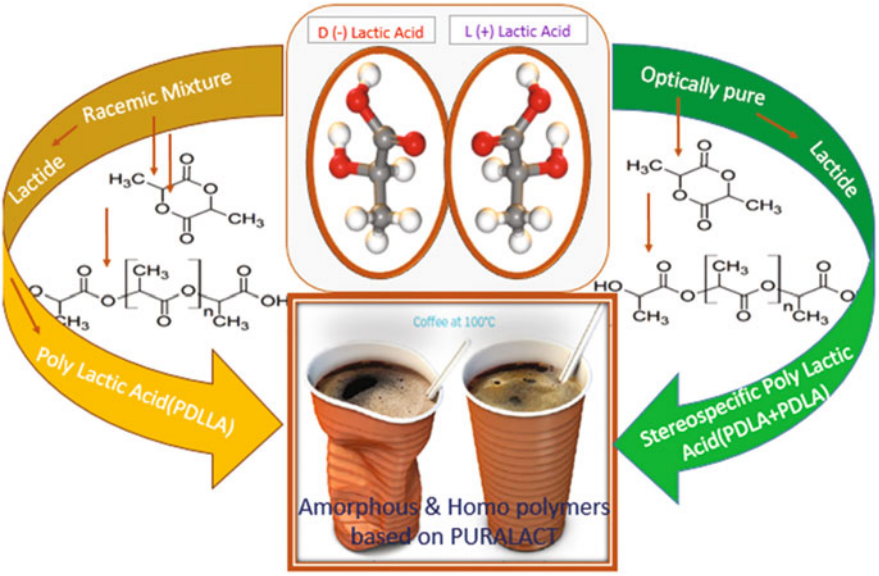

Fig. 5.4 Synthesis of stereospecific polylactic acid from optically pure monomers

D (−) lactic acid (DLA) is a versatile organic acid molecule, extensively employed in the production of thermostable biodegradable polymer, polylactic acid (PLA). Optically pure DLA is a prerequisite for the production of PLA with improved mechanical and thermal properties. D-lactic acid (DLA) is also an industrially important organic acid that has wide applications in the chemical, pharmaceutical, agriculture, textile, and leather industries. In recent years, DLA has gained a lot of attention owing to its application in thermostable biodegradable polymers production. There is increasing demand for optically pure isomers in the past two decades. According to the lactic acid and PLA market analysis report 2014, the estimated global lactic acid market in 2013 was 714,200 metric tons and is forecasted to reach 1,960,100 metric tons by 2020. The worldwide PLA production in 2013 was estimated at 360,800 metric tons and is expected to reach 1,205,300 by 2020; meanwhile, there is a need for a significant cut in the product cost almost to half of its current value ($2.2/kg). The microbial fermentation process is an attractive techno-economic strategy to produce optically pure DLA compared to a racemic mixture yielding expensive chemical synthesis. However, the choice of raw feedstock and its preprocessing requirements for microbial fermentation decides the fate of the final product cost. Hence, a low-cost feedstock with minimal pretreatment would be a suitable choice for economic DLA production. DLA can be produced through microbial fermentation by using fermentable sugars (glucose, fructose, and sucrose) and starchy materials (potato, corn, and maize) as feedstocks. However, they are utilized as staple foods in different parts of the world and could not be used as feedstock for DLA production. Alternatively, renewable, non-edible low cost, feedstocks such as cellulose, and cellulose-containing biomass (horticulture/agricultural/forest residues) could be considered for DLA production.

Therefore, the selection of a suitable raw material could significantly influence the total cost of PLA production. Industrially, DLA can be produced through either an expensive conventional chemical route using petrochemical feedstocks, which always yields a racemic mixture, or a microbial fermentation route that can produce enantiomerically pure lactic acid. In microbial fermentation, homofermentative lactic acid bacteria (HFLAB) are preferred for DLA production due to their ability the effective conversion reducing sugar solely into DLA. Microbial production of DLA is significantly influenced by the cost of raw materials. Renewable and low-cost feedstocks need to be employed for economical fermentative production of DLA and were discussed in the previously published literature. Renewable agricultural waste resources are gaining significance as potential feedstocks for the production of DLA owing to their abundant availability and low cost. Furthermore, lactic acid bacteria (LAB) growth was inhibited at high DLA concentrations as a result of the product inhibition phenomenon. However, the presence of inhibitory compounds (e.g., lignin, pectin, and recalcitrant chemicals), multi-stage cost-intensive preprocessing requirements, staple nature, and seasonal availability hinders their application in the fermentation process.

5.6 Market Demand and Stakeholders

As per the transparency market research analysis, global lactic acid market is 284,000 tons per annum in 2013 and the projected values are 375,000 tons per annum by 2020 where production technology and usage are mostly dominated by Europe, America, and the Asia Pacific regions. Lactic acid is having a wide variety of applications in the food, pharma, and cosmetic industries. Table 5.2 and Fig. 5.5 give information regarding global lactic acid volume, price ranges, and different application areas. Growing demand for polylactic acid (PLA) will reach about 1,960,100 metric tons by 2020, due to its application in various industrial spheres like biodegradable plastics, biomedical equipment, textiles, etc. Interest is more focused on Poly DL-Lactic acid (PDLLA), a hetero-polymer crosslinking between alternate D and L monomers. Growing demand for polylactic acid (PLA) will reach about 1,960,100 metric tons by 2020, due to its application in various industrial spheres like biodegradable plastics, biomedical equipment, textiles, etc. Interest is more focused on Poly DL-Lactic acid (PDLLA), a hetero-polymer crosslinking between alternate D and L monomers. This offers a 40–50 °C higher melting temperature and a variation of heat deflection temperature from 60–190 °C than either PLLA or PDLA homo-polymers alone. For this reason, there is a huge demand for optically pure DLA in the last decade. DLA is used for more specialized applications and is opening up new markets such as disposables, semi durables, and durables.

DLA can be purified to polymer grade lactic acid with improved temperature properties and is now used for not only biodegradability but also durability. An example is a television screen casing, made from PLA (using d-lactic acid—PDLA).

Table 5.2 Lactic acid production volume and cost analysis by cellulac

Product	Category	Current volume (ton pa)	Approximate price (per ton)	Segment value at a median price	Projected growth
Lactic acid	Speciality chemical or ingredient in food and solvents	320,000	$1300–$2300	$576 m	20% pa until 2016
	Specialty chemical or ingredient in bioplastic production	1,80,000	$1300–$5000	$567 m	28% pa until 2025
Polylactic acid	Substitute for fossil-based polymers	1,00,000	$2300–$6000	$415 m	28% pa until 2025

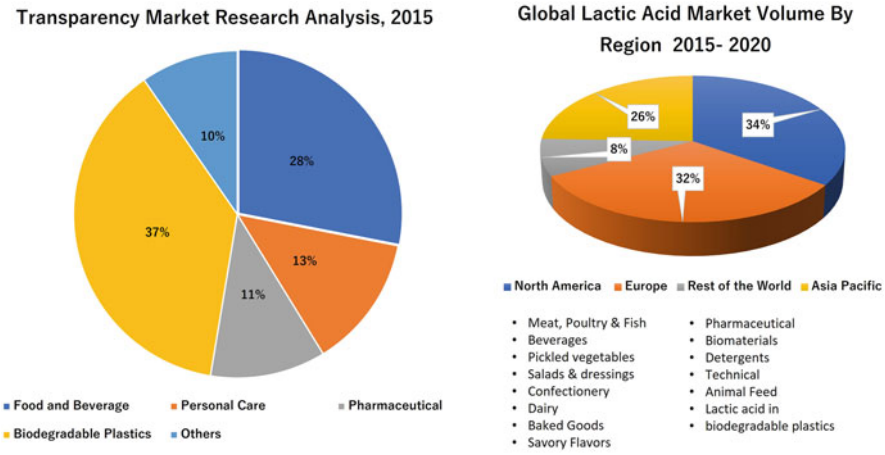

Fig. 5.5 Lactic acid production volume is distributed to different sectors and regions

Few industries are producing DLA from raw materials like corn and cellulosic materials. The raw material is one of the major cost factors in the production of bulk chemicals such as DLA. The utilization of high-energy food crops such as corn cannot serve as a suitable option for the production of PLA.

5.7 Processing Difficulty: Need of Enzymes

Globally, the utilization of sustainable feedstocks has been preferred as a cost-effective and eco-efficient strategy for value-added product synthesis. The development of an effective techno-economic strategy for the conversion of sustainable feedstock to fermentable sugars plays a key role in determining the product cost and capability to produce a variety of chemicals and fuels. Cassava (*Manihot esculenta*)

is the most widely cultivated root crop in the tropics and is the third-largest source of food carbohydrates in the tropics, after rice and maize which is grown across a broad range of agro-climatic conditions. The different stages of cassava processing involve peeling, grating, fermenting, de-watering, frying, drying which generate 25–45% of waste and are generally disposed of by land composting method. However, composting cassava fibrous waste (CFW) poses an environmental threat leading to groundwater contamination. Valorization of CFW into value-added products would be a viable option. Cassava fibrous waste is a solid waste generated during the processing of cassava tubers in sago industries in India which contains more than 50% by weight of starch and about 600–650 tons of CFW is generated in India from the sago industry. CFW disposed of cassava processing industries may cause a serious threat to the environment because of its high organic content. As CFW is rich in organic content, it can be used as cheap raw material for the production of various biochemicals. CFW is a promising raw feedstock when compared to lignocellulosic biomass because of low levels of lignin and pectin and requires minimal pretreatment. CFW has been used as a raw material for the production of various value-added bioproducts, viz. glutamic acid, ethanol, pullulan, and L-lactic acid. The first report about fermentative production of DLA utilizing CFW was attempted by our research group (Cingadi et al. 2015). While choosing a feedstock, one needs to consider the availability, conversion capability, economics of the stock, competition, and greenhouse gas savings. Considering this DLA production using CFW as a feedstock not only reduces the waste generated from the cassava starch industry but also lowers the cost of production of DLA, which is one of the major components in thermostable biopolymer PLA. A recent review by Zhang et al. addressed the advantages and consolidated the concept of CFW biorefinery.

5.8 Critical Process Parameters and Fermentation Barriers

The fermentation requires strict control of process parameters for which detailed information is needed in terms of substrate and product inhibition along with titer, optical purity, and chemical purity which will give an understanding of the range of operating conditions and parameters to run an effective production process. The temperature ranging from 35 to 45 °C and the pH ranging from 5 to 6.5 during a typical LA fermentation, a drop in pH below a critical value (due to LA production) have an inhibitory effect on the metabolic activities of the strains. In conventional operations, suitable bases and salts are added to neutralize the LA to minimize the negative effects of undissociated LA accumulation in industrial processes. However, the neutralization of LA during fermentation has major disadvantages as additional operations are required to regenerate undissociated LA from its salt and to dispose of or recycle the neutralizing cation. Calcium hydroxide is used as a conventional neutralizing agent in the fermentation reaction producing calcium lactate. Sulfuric acid is used to liberate LA from calcium lactate generating calcium sulfate as solid waste, which is currently disposed of as gypsum. Another process for the preparation

of LA without the formation of gypsum is using other neutralizing agents, like ammonia and magnesium hydroxide. When ammonia is used as a neutralizer, ammonium lactate is formed. Hence, the formation of gypsum was efficiently prevented but has the disadvantage that the formed ammonium lactate is difficult to thermally cleave to obtain the desired LA. Magnesium hydroxide can also be used to neutralize the LA, which forms magnesium lactate. Magnesium lactate is brought to react with a water-miscible organic amine to form an organic amine-lactic acid complex and magnesium hydroxide, after which magnesium hydroxide is precipitated and separated from the complex which can thermally be decomposed to liberate LA. All the extra operations and expense could be reduced if undissociated LA could be accumulated by microorganisms able to grow and metabolize at low pH levels.

5.9 Scalable Enzymatic Technologies

The imperative truth behind the biotech industries which are adopting continuous platforms over batch are the flexible operation, high productivity, and decreased cost with quality along with process integration provisions. The continuous platforms offer multiple advantages with small facilities in a simple and intensified streamlined manner. Though various technical challenges need to be addressed like homogeneity, nutrient consumption patterns, and long-term operation with sterility, genetic instability of cells which are posing the need for tight controlling strategies with effective online monitoring and control strategies. Continuous bioprocessing is preferred for lactic acid over batch production in terms of cost, flexibility, and productivity, especially with process intensification strategies where consumption of large volumes of feedstocks throughout the year is to be treated. There are reports of continuous lactic acid production coupled with process intensification by membrane-based separation (Tashiro et al. 2011). But there are very limited reports in the area of effectively controlling and online monitoring which will efficiently address the technical challenges.

The important parameter in lactic acid fermentation is the concentration of acid. High concentrations of acid are more effective for the purification of the product which reduces the cost. But high concentrations of acid are inhibiting the growth of the culture which decreases the productivity in case of non-removal of acid simultaneously. The continuous removal of acid minimizes product inhibition and favors the growth of cells, thereby more productivity. The separation of acid by membrane integration also had critical parameters in terms of cross-flow velocity and transmembrane pressure. These parameters play a key role in the separating efficiency which will have an indirect influence on the dilution rate of fermentation. All these issues can be addressed effectively by adopting good controlling strategies with effective online sensing tools which will predict the feed rates and concentrations of the product.

5.10 Purification Hurdles and Energy Accountability

The overall cost of the final product is influenced strongly by the downstream process and purification methods. Several methods have been developed by which DLA can be separated from the fermentation broth to obtain its pure form which includes neutralization and precipitation, solvent and reactive extraction, electrodialysis, membrane-based extraction, adsorption, and salting-out extraction, and molecular distillation. It is a laborious task to separate lactic acid from water although the difference between their boiling points is quite high (Komesu et al. 2015). The precipitation method is mostly preferred for industrial use while membrane technologies can be applied for easy scalability and custom-made products (López-Garzón and Straathof 2014; Choi and Hong 1999). Each of these methods has its advantages and disadvantages. Most of these methods consist of several steps, and the final yield obtained is very low. Large amounts of reagents are also required which also leads to increased cost of the final product therefore to make the process more economically viable it is necessary to develop new innovative ideas for conducting effortless separation and recovery of DLA with high yield and purity so that it can have application in bio-based refineries. Figure 5.6 gives a clear-cut understanding of DLA production process hindering parameters for assessing the possible process integration aspects further.

For the running of such biorefineries, constant power supply is necessary. Energy management is vital to keep track of the overall power consumption taking place. Equipment should be regularly monitored and improved for reduced energy consumption (Bapat et al. 2014; Joglekar et al. 2006). Very large-scale industries form energy management teams headed by an energy manager who conducts energy audits and further takes necessary actions. Another acceptable strategy for

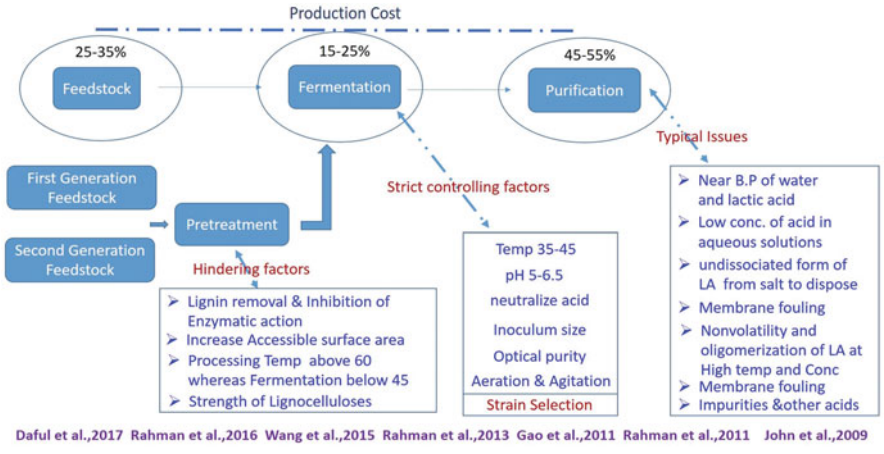

Fig. 5.6 Typical DLA production process hindering factors and cost structure of d-Lactic acid

maintaining energy supply in biorefineries is by making use of nonrenewable sources as alternatives like biogas and solar energy.

5.11 Existing Technology and Bioprocessing Strategies

D-Lactic acid has been reported to be produced by several species of lactic acid bacteria, in particular, *Lactobacillus delbrueckii*, *Lactobacillus coryniformis subsp. torquens*, *Leuconostoc mesenteroides subsp. mesenteroides, Leuconostoc mesenteroides subsp. dextranicum, Leuconostoc carnosum, Leuconostoc fallax* as well as genetically modified *Lactobacillus plantarum.* It is well known that LAB frequently require various kinds of micronutrients for its fastidious growth; however, it is expected that the LAB isolated from dairy waste would grow and produce lactic acid in the waste without the need for additional growth factors because the bacteria are living in the sludge in the first place (Nakasaki et al. 1999). Table 5.3 gives an understanding of a wide variety of microorganisms reported for the utilization of different feedstocks from renewable sources. Microbes produce a wide variety of value-added processes and capable of converting these materials, which are otherwise considered to waste, into valuable products through processes with techno-economic feasibility (Benthin and Villadsen 1995; Bustos et al. 2005; Demirci and Pometto 1992; Ishida et al. 2006; Shukla et al. 2004; Tanaka et al. 2006; Yáñez et al. 2003; Calabia and Tokiwa 2007; Ishida et al. 2006; Inaba and Maekawa 2009; Okino et al. 2008; Sangproo et al. 2012; Tashiro et al. 2011; Wang et al. 2011; Zheng et al. 2010; Zhou et al. 2003; Nguyen et al. 2012; Bai et al. 2016; Cingadi et al. 2015; Liu et al. 2014; Mimitsuka et al. 2015; Prasad et al. 2014; Reddy Tadi et al. 2017; Su et al. 2013; Zhang et al. 2016a). LAB ferment sugars via homo-, hetero-, or mixed acid fermentation. Homofermentative LAB produce lactic acid as the main product from sugars. However, the literature results highlight the need for a promising strategy for effective substrate utilization, combating salt stress, and elimination of product inhibition. In nutshell, the quest for the production of high-titer optically pure DLA in a short time with a low-cost drive is the need to formulate the proposal. There are some renowned worldwide commercial producers of lactic acid in the market, namely Purac, Galactic, Musashino, and some others which are producing based on the traditional technologies available.

There has been more emphasis on a few major challenges which has to be faced while producing DLA. The four main criteria which are required to be fulfilled are (1) purity, (2) acid tolerance, (3) carbon source, and (4) industrial parameters. Several host organisms have been rationally metabolically engineered to produce lactic acid with >99% optical purity and similarly, organisms like lactic acid bacteria which are wild and natural abilities have been modified by increasing acid stress tolerance due to which cell survivability increased at very low pH conditions enhancing overall lactic acid production. A summary of all host organisms studies used for DLA production is provided in Table 5.3 (Benthin and Villadsen 1995; Bustos et al. 2005; Demirci and Pometto 1992; Ishida et al. 2006; Shukla et al. 2004;

Table 5.3 Reported studies of D-Lactic acid in the literature on feedstocks, fermentation, and process strategies

S. No.	Organism used	Carbon source	Nitrogen source	Mode of operation	DLA titer (gL^{-1})	Optical purity (%)	r_p ($gL^{-1}.h^{-1}$)	$Y_{p/s}$ (gg^{-1})	Reference
1	*Lactobacillus delbrueckii* ATCC 9649	Glucose	Yeast extract	Batch	117.0	–	6.46	0.76	Demirci and Pometto (1992)
2	*Lactobacillus bulgaricus* Lb-12	Lactose	Yeast extract	Batch	40.9	–	–	–	Benthin and Villadsen (1995)
3	*Lactobacillus coryniformis subsp. torquens* ATCC 25600	Cellulose	Yeast extract, meat extract, and peptone	Batch	25.0	100	0.5	0.89	Yáñez et al. (2003)
4	*E. coli* W3110	Sucrose Molasses	Tryptone and yeast extract	Shake flask	51.2 48.7	>99.8	–	–	Shukla et al. (2004)
5	*Lactobacillus coryniformis ssp torquens* CECT 4129T	Glucose	Corn steep liquor, yeast extract, and peptone	Shake flask	59.0	–	0.6	–	Bustos et al. (2004)
6	*Lactobacillus delbrueckii subsp. delbrueckii* IFO 3202	Rice bran	Yeast extract, meat extract, and peptone	Batch	28.0	95	0.8	0.78	Tanaka et al. (2006)
7	*Saccharomyces cerevisiae*	Glucose	Yeast extract and peptone	Shake flask	61.5	99.9	0.9	0.61	Ishida et al. (2006)
8	*Lactobacillus delbrueckii* JCM 1148	Sugarcane Molasses Sugarcane Juice Sugar beet juice	–	Batch	107 120 84	97.2 98.3 97.6	1.48 1.66 1.16	0.9 0.95 0.88	Calabia and Tokiwa (2007)
9	*Corynebacterium glutamicumn*	Glucose	Mineral salts	Batch	120.0	99.9	4.0	0.87	Okino et al. (2008)

(continued)

Table 5.3 (continued)

S. No.	Organism used	Carbon source	Nitrogen source	Mode of operation	DLA titer (gL^{-1})	Optical purity (%)	r_p ($gL^{-1}.h^{-1}$)	$Y_{p/s}$ (gg^{-1})	Reference
10	*Lactobacillus plantarum* NCIMB 8826	Glucose and Raw corn starch	Beef extract	Batch	82.0 73.2	99.7 99.6	4.54 3.86	0.89 0.86	Okano et al. (2009)
11	*Lactobacillus plantarum* NCIMB 8826	Xylose	Yeast extract and peptone	Batch	41.2	99.2	0.686	0.89	Okano et al. (2009)
12	*Lactobacillus plantarum* NCIMB 8826	Arabinose	Yeast extract & peptone	Batch	38.6	99.9	1.42	0.82	Okano et al. (2009)
13	*Sporolactobacillus inulinus* ATCC 15538	Glucose	Yeast extract & peptone	Batch	93.4	–	–	–	Zheng et al. (2010)
14	*Sporolactobacillus* ssp. CASD	Glucose	Peanut meal	Fed-batch Single pulse multiple pulses	207 226	99.3 99.3	3.8 4.4	0.93 0.84	Wang et al. (2011)
15	*Lactobacillus delbrueckii* subsp. *lactis* QU 41	Glucose	Yeast extract, meat extract and peptone	Batch and continuous with cell recycle	86.4 20.7	>99.9	0.52 18.0	1.010 1.03	Tashiro et al. (2011)
16	*Escherichia coli* CICIM B0013–070B	Glucose	Mineral salts	Batch	122.8	–	4.32	0.89	Wang et al. (2012)
17	*Klebsiella oxytoca* M5a1	Glucose Sugarcane molasses Malto dextrin derived from cassava starch	Peptone and yeast extract	Shake flask	11–13 22–24 33–34	–	0.36–0.38 0.23–0.25 0.34–0.35	0.64–0.71 0.8–0.87 0.91–0.92	Sangproo et al. (2012)
18	*Lactobacillus coryniformis* subsp.*torquens*	Dry biomass of the microalga *Hydro dictyon reticulatum*	Yeast extract and peptone	Batch	36.6	95.8–99.6	1.02	0.458	Nguyen et al. (2012)

(continued)

Table 5.3 (continued)

S. No.	Organism used	Carbon source	Nitrogen source	Mode of operation	DLA titer (gL^{-1})	Optical purity (%)	r_p ($gL^{-1}.h^{-1}$)	$Y_{p/s}$ (gg^{-1})	Reference
19	*Sporolactobacillus laevolacticus* DSM442	Glucose	Cotton seed hydrolysate	Fed-batch	144.4	99.3	4.13	0.96	Li et al. (2013)
20	*Lactobacillus delbrueckii* subsp *lactis* ATCC 4797	Casein whey permeate	Casein hydrolysate	Batch	24.3	>98	0.38	0.49	Prasad et al. (2014)
21	*Escherichia coli* HBUT-D	Glucose	Yeast extract	Batch	127.0	99.5	6.35	0.93	Liu et al. (2014)
22	*Sporolactobacillus inulinus* Y2-8	Corn flour hydrolysate	Yeast extract	FBB-batch FBB-fed-batch	145.8 218.8	99.0	1.62 1.65	0.96 –	Sun et al. (2015)
23	*Lactobacillus delbrueckii delbrueckii* NBRC 3202	CFW hydrolysate	Yeast extract	Batch	16.15	0.5	0.9	98.05	Cingadi et al. (2015)
24	*S. cerevisiae* NBRC 10505	Cane Sugar	Peptone and yeast extract	Continuous	46.6–52.1	99.9	7.1–8.1	0.54	Mimitsuka et al. (2015)
25	*Sporolactobacillus inulinus* NBRC 13595	Palmyra palm jaggery	Whey protein hydrolysate	Batch	189.0	>98	5.25	0.94	Reddy Tadi et al. (2017)
26	*Sporolactobacillus inulinus* YBS1-5	Corncob residue	Cottonseed meal	Fed-batch	107	99.2	1.19	0.85	Bai et al. (2016)
27	*Lactobacillus plantarum* NCIMB 8826	Corn stover	Soybean meal extract	Fed-batch	61.4	>99.0	0.32	0.77	Zhang et al. (2016a)

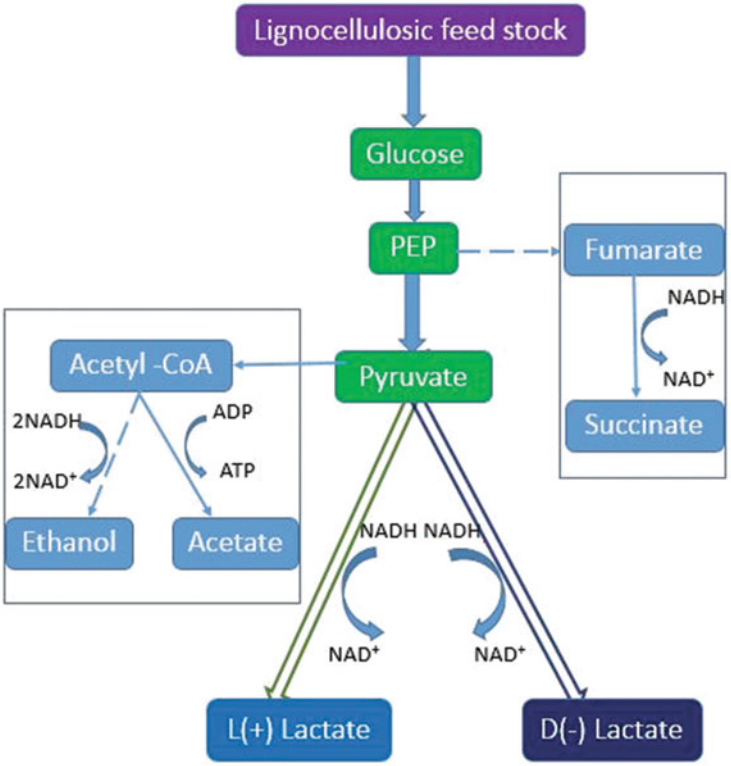

Fig. 5.7 Lactic acid pathway: From lignocellulosic feedstock to D-Lactic acid

Tanaka et al. 2006; Yáñez et al. 2003; Calabia and Tokiwa 2007; Li et al. 2013; Inaba and Maekawa 2009; Okano et al. 2009; Okino et al. 2008; Sangproo et al. 2012; Tashiro et al. 2011; Wang et al. 2011; Wang et al. 2012; Zheng et al. 2010; Zhou et al. 2003; Nguyen et al. 2012; Bai et al. 2016; Cingadi et al. 2015; Liu et al. 2014; Mimitsuka et al. 2015; Prasad et al. 2014; Reddy Tadi et al. 2017; Su et al. 2013; Sun et al. 2015; Zhang et al. 2016a).

CFW is enriched in starch which needs to be enzymatically converted to glucose at the first step. This glucose gets converted to DLA in the second step through microbial fermentation. The existence of lactic acid bacteria can be considered wealth in nature due to its large number of applications. Amylolytic lactic acid-producing bacteria are capable of doing the conversion from starch to lactic acid directly through a single step. But the whole metabolic process becomes an overburdening task for the organism due to which the end product yields and productivity decreases and is not considered suitable for industrial-scale applications. Analogously, homofermentative lactic acid bacteria (Fig. 5.7) in general will be more preferred for the production of DLA from enzymatically hydrolyzed sugar through the Embden Meyerhof pathway (EMP), and the end product obtained is

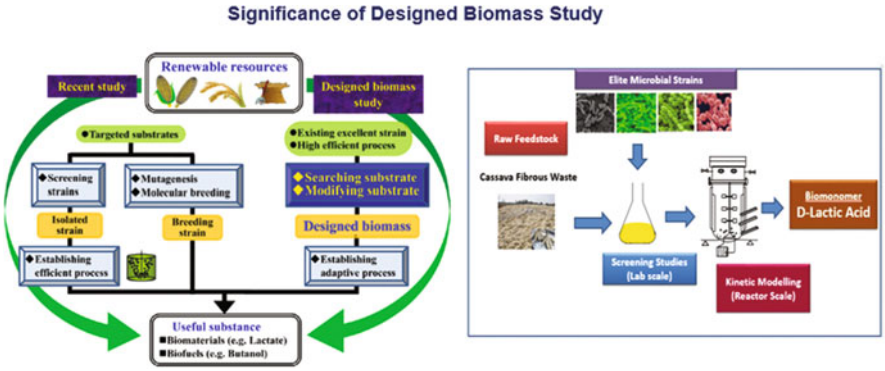

Fig. 5.8 Designed biomass approach and selection of elite strain for fermentation from renewable feedstock

more efficient and of high optical purity which is highly essential for PLA synthesis. Apart from that, it is also necessary for the genetically manipulated (GM) host organisms to maintain stability throughout without getting mutated and also not cause any harmful outcomes in nature, so that it can be highly recommended for commercial use. This approach is unsure in terms of GM systems. There is an alternative approach in terms of the designed biomass study for this case which is represented schematically in Fig. 5.8 for better understanding.

5.12 Summary

There exists a vast scope of cassava in all developing countries including India. Cassava being a carbohydrate-rich crop has a huge potential to improve the economic structure of a nation if properly executed. With more efforts in R & D, improvements can be made in the agricultural sector. Limitations associated with cassava can be sorted out by trial and error. Genetic engineering is now a boon to society and has huge scope in multiple areas of study. With genetic engineering techniques, new hybrids of wild cassava can be made with improved properties like drought resistant, disease resistant, high yield, increased shelf life post-harvest, etc. Cassava mosaic disease is a very common disease associated with cassava cultivation and can be prevented by manipulations done at the genome level along with enhancement in starch and fiber content. DLA is one of the versatile organic acids that needs more attention from a research point of view due to its emerging applications in the polymer industry. There is also an urgent need to discover more viable feedstocks for the production of DLA by biological means. Starch extraction technologies need to be developed along with the modernization of traditional equipment being used in the sago industries. Sago industries are still

emerging, and the marketing of cassava-derived foods has to be improved globally. Arrangement of national symposiums will create more awareness about cassava and its derivatives and will also bring limelight to its potential aspects. New government policies have to be made which will promote cassava cultivation and will lead to the foundation of a greater number of biorefineries. Bioprocessing equipment has to progress for the efficient downstream processing and marketing of optically pure DLA at a much cheaper price. Once the whole manufacturing plan is established and optimized, biorefineries will serve as a stepping-stone for commercial-scale production. Although literature studies show different ways to produce DLA from various substrates using a wide variety of host organisms, not all are able to successfully reach the industrial level for commercialization. To scale up these scientific studies, optimization of the process needs to be done on a large scale and this can be only achieved if we are able to visualize the bigger picture first and then start working from scratch on a bench scale. The partnership between research institutes and the private sector is the best way by which we can promote this and attain success.

Competing Interests The authors declare that they have no competing interests.

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Chapter 6
Waste Derived Supports for Immobilization of Lipase Towards Enhancing Efficiency and Reusability of Enzymes

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Abstract The biorefineries plays vital role in maintaining energy symmetry which is a critical factor that controls the socio-economic development of any nation. Enzymatic catalysts are preferable because it has capacity to change over triglycerides along with free oleaginous acid within a single move, and easy product separation with recovery at a minor proportion of oil to alcohol minimizes aftereffects and unwanted substances. So, this chapter introduces the proper lipase immobilization methods and technologies onto indigenously prepared various supports like silica, carbon, zeolite, and magnetic nanomaterials to overcome the demerits like moderate reusability, higher cost and lesser activity. Additionally, highlights are cost optimization of catalyst synthesis processes using wastes utilization by applying advanced statistical tools such as response surface methodology, Taguchi method, etc. that provides higher surface area for enzymatic reactions. Thus, waste valorization towards support synthesis for lipase immobilization could help in the advancement of biorefineries.

Keywords Lipase immobilization · Reusability of enzymes · Support · Biomass valorization

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Abbreviations

APTES	3-aminopropyl triethoxy silane
CLEAs	Cross-linked enzyme aggregates
CNTs	Carbon nanotubes
DAG	Diacylglycerol
EA	Enzyme adsorption
EAC	Enzyme adsorption, cross-linking
EAPC	Enzyme adsorption, precipitation, and cross-linking
FAAE	Fatty acid alkyl ester
FAEE	Fatty acid ethyl ester
FAME	Fatty acid methyl ester
FFA	Free fatty acid
GOD	Glucose oxidase
HPAs	Heteropolyacids
HPR	Horseradish peroxidase
HR-TEM	High resolution TEM
IS	Ionic silsesquioxane
MAG	Monoacylglycerol
MNPs	Magnetic nanoparticles
MOF	Metal-organic frameworks
MPTMS	3-mercaptopropyl trimethoxy silane
MX	Magnetic xerogel
NOx	Nitrogen oxides
NPs	Nanoparticles
PANFs	Polyaniline nanofibers
PEI	Polyethylenimine
SENs	Single-compound nanoparticles
Sp1	Specificity protein 1
Sp2	Specificity protein 2
Sp3	Specificity protein 3
TAG	Triacylglycerol
TEM analysis	Transmission Electron Microscopy analysis
TG	Triglyceride
TLL	*Thermomyces lanuginosus*
USD	US doller
XRD	X-ray diffraction
ZIF 67	2-methylimidazole cobalt salt

6.1 Introduction

Administrating biomass-derived industry particularly in precarious circumstances over the years is a real challenge both from process development strategies and from a market point of view.

The theory of utilizing vegetable oil (particularly, experimented on neat peanut oil) as liquid fuel in internal combustion engine was pioneered by the great German inventor, Rudolf Diesel in the year 1900 to figure out the consequences of country-side agricultural people in France, those who have endured periodical shortfall of petroleum derivatives (Knothe 2001). In the late 1800s, a few dangerous and troublesome steam engines were replaced by his eminent creation "Diesel Engine" which was authorized by the French government (Shay 1993).

The vegetable oil mostly comprises of free fatty acids (FFA; 1–5%), monoacylglycerides (MAGs), diacylglycerides (DAGs), triacylglycerides (TAGs; 90–98%) along with tocopherols, phospholipids, carotenes, phosphatides slight sulfur component, water as well as the fatty acid composition of TAGs contains linoleic acid (C18:2), stearic acid (C18:0), palmitic acid (C16:0), linoleic acid (C18:3), oleic acid (C18:1) (Goering et al. 1982) and direct injecting neat plant oil as fuel causes a significant engine complication because of their low volatility along with high viscosity (Mether et al. 2004). Within the period from the 1900s to 1930s, pyrolysis of triglyceride was the only possible process to which nations like Belgium, China, Brazil, and North America contributed their effort endlessly.

Concurrently, in 1937, the concept of fatty acid alkyl ester (FAAE), a long-chain fatty acid with short-chain alcohols which is an outcome of plant oil transesterification was first introduced by Belgium inventors. Later on, animal fats, waste fats, and residuals were certified as alternative biodiesel resources. The attributes of FAAE are higher flash point, pour point, cloud point, cetane numbers along with greater viscosity, lower sulfur content, and high oxygen content reducing the heat of combustion (Peterson and Jose 1995). Where, considering fatty acid ethyl ester (FAEE) on top of fatty acid methyl ester (FAME) because of its higher energy content with cetane number as well as poor cloud point, pour point with density along with FAEE exhibits a promising fuel composition that reduces smoke densities with nitrogen oxides (NOx) (McCormick et al. 2001).

In the present world, increasing consciousness on biowaste could serve as a treasurable feedstock for integrated biorefinery is needed. The word "Valorization" explains the transformation of biowaste into energy, fuels, and creating value-added products. It is also embedded in the scientific area to sustain the new profit-making economy.

More or less all production processes that come under biorefinery are dealing with several constraints in spite of various promising prospects like lipases are relevant biocatalyst for transesterification of high FFA and moisture containing biomass. Additionally, lipase requires average reaction conditions, uses broad substrate range, acts as inert in presence of water and acid, helps in easy product separation, provides high probability of regeneration and reuse of immobilized remnant.

In brief, lipases originated from all living organisms dispersedly obtainable in microorganisms (mostly in bacteria, yeasts, and filamentous fungi), plants (mostly in castor seed enzyme, papaya latex, and oat seed enzyme), pancreatic lipases are available from animals. In general, they are categorized as extracellular (immobilized enzyme) and intracellular lipase (immobilized whole cell). The specification of lipase has been categorized into stereo-chemical, lipid class, fatty acid along with positional combinations. Microorganism-rich lipases are also inscribed as "Silver Bullet" due to their unique attributes like substrate specificity along with its selectivity, regioselectivity, and enantioselectivity. Though, there are numerous affecting factors, i.e., pH, enzyme activity, and substrate concentration, moreover, immobilization provides optimum conditions for lipase to perform skillfully.

Nevertheless, lipases are sensitive in presence of alcohol (remarkably, in the case of the most common acyl acceptor, methanol). To maintain the stability of the enzyme, the solution is to control the concentration of alcohol followed by stepwise reaction or by enzymatic immobilization which works as a shield around the lipase to protect it from solvents like alcohol and provide resistance to thermal activation along with it removing downstream operations like separation and recycling.

Enzyme immobilization follows two distinctive conformations based on the situation of the polypeptide chain (commonly known as the lid), i.e., inactive closed form (blocked lipase activation site) and active open form (exposed lipase activation site). Few familiar methods are employed for lipase stability those are cross-linking as well as covalent bonding. Among them, covalent coupling with insoluble matrices has proven beneficial for the adsorption and entrapment process. Thus, immobilization is a profitable approach to get more residual activity and provides easy regeneration of lipase.

Though immobilization is a high-priced process in spite of that it may change the function and shape of lipase or in a few cases, enzymes may disconnect. To decode the condition, a few advanced methodologies including magnetic particle carriers, protein-coated micro-crystals, and electrospun nanofibers with cross-linked protein-covered micro-crystals have been adopted.

Nanoscience has the ability to show its endeavor in enzymatic immobilization to get optimum biodiesel yield by protecting from steric hindrance. The employment of nanomaterials is visible in the bioindustry and those are nanoparticles (NPs), nanotubes, nanofiber, nanopores, nanosheet, nanohorns, and nanocomposites in addition to several nanometals like gold NPs, nanosilica, zirconia NPs, nano diamonds, nano graphenes. As nanoparticles have their distinctive properties and those are a high proportion of surface area to volume, maximum porosity along with surfaces can be easily modified (Agrawal and Verma 2021; Goswami et al. 2020). Activated carbon is generally obtained from charcoal which is contemplated as porous support material for over a decade.

This manuscript is a compact effort to present concepts like enzyme implementation on top of other catalysts, expanded scope of enzymatic immobilization, and enzyme entrapment using carbon nano-support.

6.2 Lipase: Action Mechanisms

Lipases also termed as triacylglycerol ester hydrolases (EC 3.1.1.3) and clarified as hydrolyzed serine alongside a working site conveying histidine serine with aspartate containing amino-corrosive group (Melani et al. 2019). Lipases are obtained from plant tissues, animals, and also microorganisms. Such natural sources explore its dynamicity with a broad scope of having strain flexibility, temperature, pH, explicitness, and hydrolysis rate (Maldonado et al. 2016). In non-aqueous mediums, lipase displays an impartial pH range with great steadiness. Hence, stability expands with quality immobilization.

In non-aqueous conditions, lipases act as a catalyzer in the transesterification, inter-esterification, and esterification processes. At the interface, lipases hydrolyze triacylglycerols in the presence of non-aqueous fluid; however, rest of the proteins may likewise involve the combination of long-chain unsaturated fats with esters from alcohols at a low dampness climate (Maldonado et al. 2016). In the transesterification process, for the age of unsaturated fat methyl esters, lipase mostly admires a two-venture system that follows the most of Ping-Pong Bi-Bi mechanism (Al-Zuhair et al. 2007).

Most triacylglycerol lipases are regiospecific that enlighten few facts like easy essential ester bond hydrolyzation at its sn^{-3}, sn^{-1} positions, outer situations inside the triacylglycerol, and can create possibly one free unsaturated fat and diacylglycerol, or two free unsaturated fats and 2-monoacylglycerol that remain unhydrolyzed. Normally, from Bacillus sp. extracellular bacterial lipases are obtained (Sugihara et al. 1991). Monoacylglycerol lipases (EC 3.1.1.23) catalyze the hydrolysis at the particular sn^{-2} situation of 2-monoacylglycerol into free unsaturated fat and glycerol. Such lipases might be available in the protein extricate and veiled when estimating movement with standard action strategies like those dependent on triolein hydrolysis estimation (Lanser et al. 2002).

Monoacylglycerol lipases have been the object of not many investigations (Tsurumura et al. 2014), despite the fact that they may be available in some microbial enzyme arrangements (Li et al. 2020). Different lipases are vague and can follow up on any of the ester obligations of the triacylglycerol and in this manner separate the triacylglycerol to deliver free unsaturated fats and glycerol as the eventual outcomes. This is the situation of lipases from *Staphylococcus aureus* and hyicus (Davranov 1994), Corynebacterium acnes, *Geotrichum candidum*, *Chromobacterium viscosum,* and *Penicillium cyclopium* (Jaeger et al. 1994). Another option for monoacylglycerols hydrolysis is the acyl movement in the glycerol spine from the sn^{-2} situation to sn^{-1} or sn^{-3} positions (Wei et al. 2010). The particularity of lipases relies upon the length of unsaturated fats, presence of twofold securities, stretched gatherings, and, thusly, response rates might have significant varieties relying upon the triacylglycerols synthesis available in fat waste. Lipases are particularly dynamic against long to medium-chain unsaturated fats, generally obtained in animal fat waste (Toldrá-Reig et al. 2020).

The functionalized alteration of familiar ferromagnetic resources like Fe_3O_4, γ-Fe_2O_3, Fe, Co, and Ni (Tronc et al. 2000; Jana et al. 2004) assists to hold on to the magnetic properties and reusability, as well as rapid and trouble-free segregation of biocatalyst accompanied by an external magnetic field, at the same time sustaining the admirable catalytic execution also the specific hydrophobic properties, pore size, surface area with recyclability which substantiated by the principles of green chemistry that acts to control waste formation.

6.3 Supports for Enzymes

The connotation of support substance is stuff that is utilized to functionalize a catalytically dynamic element at its exterior portion to execute the role of catalysis. It serves the well-built concentration of reactant-catalyst holding sites that assist to distinguish from conventional heterogeneous catalysts, at the same time as keeping up with the benefit of trouble-free exclusion from the reaction blend. To boost and execute the catalytic action in biodiesel manufacturing processes by facilitating the utilization of inexpensive natural resources with high FFAs, numerous current studies have been issued on surface alteration (impregnation) by means of catalytically dynamic entities including lipase enzymes, Lewis acids, or bases, metals, or metal oxides are mostly obtainable supports of synthetic, organic, and inorganic in nature to bring to a halt over the fulcrum substance to create heterogeneous biocatalysts (Toh-Ae et al. 2014; Radoman et al. 2015).

Nanocatalyst composites are formulated with physical including laser thermal cracking, pulse volatilization settling, high-energy ball milling, inert gas fixation, melt integration, flash spray thermal cracking, and electro-spraying chemicals such as hydrothermal, sol-gel, microemulsion, chemical vaporization, and polyol and genetic such as microbes served by bio templates assisted biosynthesis, biosynthesis, and greenery extracts served by biosynthesis techniques. The execution of nanocatalysts examination takes place by employing magnetic catalysts (Ghalandari et al. 2019; Ambat et al. 2019; Gardy et al. 2018), supported metal oxides (Montero et al. 2010), alkaline earth metal oxides (Zhao et al. 2013). Composites acquired from trash biomass like carbonaceous (from greenery wastes such as husks), calcareous(from sea animal shells, eggshells, waste marine barnacles or calcareous rocks), siliceous(from sugarcane bagasse, bamboo leaves, wheat straw), and various minerals attained from dolomite, kaolinite, calcite, and bauxite may differ in features like dissimilar arrangement of particle dimensions, profile, pore construction with pore volume, hydrophobic nature, and density and have to be unwavering to physical, chemical, and microbial decay (Zhao et al. 2013; Bhardwaj et al. 2020; Kumar et al. 2020). Porous supports by means of controlled pore distribution are incredibly fascinating for lipase immobilization as they put forward a broad exterior region and consequently, maximum enzyme loading. Nevertheless, discretion has to be audited when miniature pores might get chocked by lipase, dropping its ability. To defeat the mentioned difficulty, composites having attributes like extensive pore magnitudes with an improved surface area provide finer accessibility.

6.3.1 Mesoporous Nano-support

Mesoporous nano-catalyzer enraptured huge regard for help reactants dispersion into the center of the catalyzer's acidic locales to dynamic locales. Since the disclosure of porous materials during the 1990s, different techniques have been made in the arranging and progression of such materials with phenomenal surface properties, for instance, pore dimension, stability, and dynamic areas to chip away at their show in various responses. The pore dimension does not exceed the range of 15–300 Å reliant upon the combination strategy and related cooperation among antecedent and format particles (Melero et al. 2006). Mesoporous nanomaterials mainly extended to classifications like ordered (like ordered mesoporous carbon and silicate are more preferable due to homogeneous allocation of pore dimension with massive pore volume, extensive surface dimension, ideal mass transfer properties, controllable wall synthesis, greatest thermal with mechanical durability) and non-ordered by coordination with the demonstration by IUPAC (Wan et al. 2007). Among them, requested mesoporous have been for the most part pondered.

In general, the reactant particles, for example, TG (5.8 nm) assists by the enormous pore dimension, to penetrate the pore channels and reach the dynamic destinations (Mbaraka et al. 2006; Wu et al. 2014). A number of essential synthetic processes for constructing mesoporous elements are microwave, polymeric precursor, sol-gel, and hydrothermal (Song et al. 2018; Xie and Zhang 2016). Direct synthesis like co-condensation reactions with a post-synthesis process like grafting process assists to fabricate meso structured nanocatalysts. In the existence of the structure-agent template, the direct synthesis of organosiloxane (mercaptopropyl trimethoxy silane) in addition to siloxane (explicitly tetramethyl orthosilicate or tetraethyl orthosilicate) acts as an ambassador. Such post-synthesis defines as an alteration method of the surface comprised of organic forerunners in presence of silica that is 3-mercapto propyl trimethoxy silane (Soltani et al. 2017).

Be that as it may, basic activity misfortunes have been represented by immobilized lipase impetuses, which can be given out to the unique site opposing, the mass exchange impediment, or the impetus structure alteration during immobilization measures (Zhang et al. 2019). Thus, it is at this point testing to cultivate lipase-based catalysis systems with high reactant activity for biodiesel readiness. By stacking lipase into supporting materials with a hydrophobically changed surface, the hydrophobic cooperation prompts a plan change of lipase iotas in which the top moves (McKendry 2002; Dodds and Gross 2007), subsequently the powerful regions become accessible and the reactant activity of immobilized lipases is chipped away at appeared differently in relation to the free ones (Demirbaş 2001; Li et al. 2016). In 2015, Mathesh et al. analyzed lipase development as a segment of surface hydrophobicity of lessened graphene oxide maintains and saw a hyper-commencement property (B2.2 times higher activity than that of free lipase) for most hydrophobic backings (Raheem et al. 2015). Be that as it may, versatile applications using complex lipase/graphene papers achieved an enormous activity decrease (B40%), basically due to a high mass trade obstacle (Raheem et al. 2015).

Recently, our social event declared the mix of hydrophobic mesoporous silica nanoparticles with tunable pore sizes as lipase maintains and showed unparalleled displays with 5.23 events higher activity than the free impetus and astonishing steadiness (Salama et al. 2017). Nevertheless, the little particles size (B60 nm) and the high arranging cost limit their application in viable catalysis reactors, as ceaseless stream gadgets. These bad marks have been fundamental concerns for the hyper-enacted lipase catalysis systems.

6.3.1.1 Carbon Support Preparation

Catalysis can be credited to their hitting ascribes related to textural, conductivity, strength, and hydrophobicity. They have investigated their capacities in various fields, for example, as impetus support and as unique stage in catalysis, as the anode in energy holding devices, in-water cleaning, in gas segregation, and as soil added substance. Carbon can show three states of hybridization, explicitly sp, sp^2, and sp^3, which drive the plan of particular pentagonal, hexagonal, or heptagonal structures (Lu et al. 2012). These "enchantment" plans license the improvement of an arrangement of commended carbon materials, similar to fullerenes, carbon nanotubes, graphene, and mesoporous carbon. But these materials solely involve carbon particles, they have extraordinary properties due to the phenomenal provisions owing to the amazing design of carbon molecules through an adaptable calculation. Set up analysts have seen the meaning of carbon materials with two elevated Nobel prizes: (1) fullerenes—1996 (Chemistry, Prof. Robert F. Curve Jr., Prof. Harold W. Kroto, and Prof. Richard E. Smalley) and (2) graphene—2010 (Physics, Prof. Andre Geim, and Prof. Konstantin Novoselov) (Lu et al. 2012; del Monte et al. 2015).

6.3.1.2 Activity of Carbon as Nanomaterial

Present investigations mirror the carbon materials that have thrived as great sans metal dynamic stage impetus upholds a few biomass valorization responses (Pérez-Mayoral et al. 2016). Due to having such characteristics as huge explicit surface region, tailorable permeable designs and surface science, superb synthetic security in corrosive or base media, surprising aqueous dependability, and proficient functionalization, they show a wide assortment of range. Numerous sorts of conventional carbon assets, such as actuated carbon, carbon dark, smooth carbon, pyrolytic carbon, and polymer-inferred carbon have been used for offsetting reactant dynamic stages. Owing to the high unequivocal surface region and rich surface science, these carbon materials license the course of action of outstandingly dissipated metal particles (Pd, Ru, Ni, Cu, Ag, Fe, etc.) all through the impetus system, achieving progressed protective nature from sintering even at higher metal loadings at raised temperature conditions (Zhu et al. 2015).

6.3.1.3 Carbon Nanotubes (CNTs)

Carbon nanotubes (CNTs) are depicted by a hexagonal arrangement of sp^2 carbons that have constraints over depression computations. CNTs are acquired in two structures as single-walled and multi-walled CNTs reliant upon the amount of carbon layers present in the cylindrical divider. Single-walled CNTs are semiconductive with breadths of around 0.4–2 nm, however, multi-walled CNTs are metallic (Georgakilas et al. 2015). Curiously, the openings of CNTs can limit the absolute of dynamic metal NPs during impetus responses (Zhang et al. 2015). Graphene is a two-dimensional material involving a single layer of carbon particles in hexagonal sp^2 hybridization (Zhu et al. 2015). Two kinds of C–C bonds, to be explicit in-plane s-bond and out-of-plane p bonds are generally found in graphene. The electronic properties of graphene, obliged by out-of-plane p-bonds, accept an imperative part in working on the collaborations of graphene with metallic NPs, and along these lines the synergist execution of graphene in biomass valorization. Mesoporous carbons, mesostructured carbon-based composites, and carbon nanofibers have moreover gotten inconceivable thought for reactant biomass reorganization.

Deformative destinations melded into the sp^2 arrangement of CNTs, graphene, or established carbon can insistently affect the surface components and synergist efficacies (Liu et al. 2015). Heteroatom doping is an appealing procedure, which takes advantage of flawed structures in carbon materials (Patel et al. 2016). A selection of parts, including nitrogen, phosphorus, boron, and sulfur have been viably doped into carbon materials.

Unusually, constructional and electronic components of doped carbon materials can be broadly exceptional according to the perfect analogs, due to the synergistic host–dopant affiliations. For instance, doping with electron-rich nitrogen can change the bend thickness and the charge apportionment of carbon particles. This will provoke improvement of p-restricting possibility and further created basicity on the carbon surface (Yang et al. 2017).

Additionally, N-doping can help the plan of new surface nucleation destinations and different powerful focuses on the N-rich locales (Chen et al. 2014). Curiously, boron doping produces destructive destinations in carbon materials. The presence of such practical locales on the carbon surface does not simply add to additional created joint efforts between the carbon support and the dissipated metal NPs, yet moreover enables the usage of carbon materials as able without metal impetuses.

To furthermore deal with the reactant development and selectivity of awesome or doped carbon materials, the immobilization of various corrosive (nitro, sulfate, or phosphate) or base functionalities ($–SO_3Na$, –COONa, –ONa, or $–NH_2$) on the carbon surface has been finished using fitting blend strategies (Ali et al. 2017). Anchoring of corrosive practical gatherings on the carbon surface can occur through strong C–S, C–C, and C–O bonds. Base functionalized carbons have similarly been found wonderful reactant efficiencies, for example, in biodiesel readiness (Tessonnier et al. 2009). Be that as it may, these functionalized carbons experience

the evil impacts of a couple of hindrances, especially by virtue of destructive social affairs. For example, destructive bearing carbon materials are typically prepared by sulfonation of somewhat carbonized normal molecules using especially thought perilous liquid acids, for instance, H_2SO_4 or HNO_3 (Liu et al. 2016). These show ominous effects on the plan similarly to the steadfastness of carbon materials, which in this manner could provoke inefficient recovery of the stimulus from the reaction mix.

The numerous possible plan of action to construct novel catalysts accompanied by mini particle range with maximum activity has been furnished using advanced nanochemistry (Shylesh et al. 2010). Though, the filtering of nanocatalysts segregation along with recuperation is not feasible as a result of zero detention of tiny nanoparticles over the sieve. On the other hand, such troublesome recuperation routes relating to centrifugation from the system of reaction as well as indisputably time taking, energy-inefficient as well as expensive are not applicable for industrial purposes.

6.3.2 Magnetically Active Nano-Support

Magnetically active significantly formulated support materials are also being examined as their magnetic attribute permits the efficient exclusion and regeneration of the catalyst, which might simplify by eliminating the centrifugal process and ultrafiltration methods of such catalyzer that handles commercial sorting out of catalyzer. Fabrication of nanoparticles having magnetic properties in a broad array is applicable in the preparation of biodiesel from inexpensive raw materials. In the middle of them, iron oxide (Fe_3O_4) and heteropoly acids (HPAs) nanomaterials might get fascinated by an excellent treaty of interest owing to their acidity, trouble-free separation along with recyclability. The HPAs have been categorized into three classes and those are hierarchically classified in the tertiary, secondary, and primary structural configurations of HPAs (Mizuno and Misono 1998).

Distinctively, magnetic nanoparticles consist of pure metal (like Co, Fe), alloy-based (like FePt, $CoPt_3$), iron oxide (like -Fe_2O_3, Fe_3O_4), along with spinel-type ferromagnet (like $MnFe_2O_4$, $MgFe_2O_4$, $CoFe_2O_4$) (Bilal et al. 2018).

Excellencies like non-toxicity with biocompatibility encountered by magnetic nanoparticles (MNPs) but have a tendency of oxidation followed by accumulation. In addition, customization of exterior periphery takes place with the help of a cross-linking agent to attach the enzyme. The most effective approach is a silica casing created over the exterior periphery with the help of an amino-functional reagent. In presence of 3-mercaptopropyl trimethoxy silane (MPTMS) or 3-aminopropyl triethoxy silane (APTES), mesoporous silicon encapsulated to customize Fe_3O_4 nanoparticles afterward glutaraldehyde helps to link up lipase like *Burkholderia* sp. through the customized support of Fe_3O_4 nanoparticles (Christopher et al. 2014). Such customization provides an improved biodiesel yield of 90% (Tran et al. 2012).

An additional approach is to protect MNPs using organic polymers, as well as biopolymers with synthetic polymers where enzymes are easily entrapped by a polymer shield over the exterior periphery of MNPs in presence of numerous functional groups. The formulation of Fe_3O_4 MNPs can be either obtained by polymer assimilating pioneer solution or outwardly core casing construction (Bilal et al. 2018). As covalent attachment assists magnetic chitosan in lipase entrapment (Xie and Wang 2012). Nanobiocatalysts separation from an oily system is a bit tricky although the MNP's magnetic features assist in enzyme segregation from the reaction medium (Ngo et al. 2013). This approach may lead to the reaction at instant completion moreover enzyme employment may bring a further application (Bilal et al. 2018).

6.3.2.1 Magnetic Nanoparticle Stabilization

The metal or metal oxide magnetic nanoparticles are for the most part shaky and also oxidize or hydrolyze promptly in the case in presence of air, they are corrosive for a more extended time frame, individually. More critically, they agglomerate quickly, shaping bigger particles that truly influence the readiness of ensuing impetuses. In absence of a stabilizer, high-energy metal nanoparticles can meet into bigger particles during the catalytic reaction. This, thusly, prompts the deficiency of dynamic locales and explicit surface regions, hence fast impetus deactivation (Lewis 1993) Subsequently, to successfully control the molecule development of MNPs just as to keep up with their nanosized-dependent trial, they should be covered with natural or inorganic substrates. For example, the magnetic Fe_3O_4 nanoparticles were covered with oleic acid to make them steady and will in general get monodispersed nanoparticles (Zillillah et al. 2012).

6.3.2.2 Functionalization over Magnetic Nanoparticles

The attractive impetuses with various reactant properties can be blended by immobilization of corrosive, antacid, and catalyst practical species onto covered attractive particles.

6.3.2.2.1 Impregnation Method

Usually, the impregnation method is defined as to plunge the carrier within the blend consisting of the dynamic element initially, eliminating the unnecessary fluid after accomplishing the equilibrium, afterward the blend passes through a few techniques like drying, calcination, and activation to get the magnetic catalyst (Si et al. 2005). The plunged dynamic element within the blend must contain the qualities like high solubility, stable structure as well as decomposition within unwavering genus at a certain temperature, like nitrate, ethylamine salt, amine salt, and others. By applying

this method, the mostly acquired magnetic nanocatalyst is KF/CaO-Fe_3O_4 (Hu et al. 2011). Initially, the blending of Fe_3O_4 with oxides (MgO, CaO, or SrO) by maintaining homogeneity afterward the absolute plungation of the blend within a different mass of KF aqueous solution along with drying and at last, the calcining desiccated substance at 300–800 °C provides the magnetic nanocatalyst. Dissolving into the KF aqueous solution gives a better result of CaO as well as a unique porous structure of magnetic nanocatalyst. Though the impregnation method is very effortless and economical, it has a few demerits such as poor recyclability and easy separation of catalyst active sites due to weak interactions with support, which limits its future application.

6.3.2.2.2 Coprecipitation Method

Being a low-cost, scalable, and simple process, the coprecipitation method gets prioritized in numerous research works. The fundamental theory is defined as dissolving precipitating agents into aqueous solutions that carry metal salts provide formations like hydrated oxides, crystals, or gels of carbonate. Later on, the obtained catalyst is washed, dried, and at the required temperature calcined after precipitate separation.

By applying this method, the mostly acquired nano-magnetic solid base catalyst was obtained by filling up CaO over the exterior surface of Fe_3O_4 via Na_2CO_3 along with NaOH as precipitants (Liu et al. 2010). The direct filling of CaO on Fe_3O_4 acts as an efficient shield for the magnetic core along with providing a huge amount of dynamic sites. The choice of precipitant and the ratio of CaO with material portrays as a key parameter that influences the future exercise of the precipitation method. Due to the quick synthesis of magnetic catalysts, the present work focuses on such favorable processes as the coprecipitation method that is generally employed for the preparation of CaO at ($Sr_2Fe_2O_5$-Fe_2O_3) (Zhang et al. 2016), and CaO at Fe_3O_4 (Ali et al. 2017).

6.3.2.2.3 Hydrothermal Method

The hydrothermal state is defined as the presence of water at a high operating state such as temperature or pressure, and a few distinctive qualities like positive diffusion along with sizeable crystal formation make the acquired substance so distinctive. The operating condition like temperature within 150 °C defines as low-temperature hydrothermal synthesis which is incredibly advantageous as it provides larger pore sizes having configurations like non-equilibrium metastable phase, also high-temperature hydrothermal synthesis while the temperature is higher than 150 °C. And at 200 °C, magnetic nanoparticles can be obtained without merging surfactant but escorted by the help of $CoFe_2$-$xGdxO_4$ (where, $x = 0–0.25$).

XRD study displays the pure phase of acquired fine particles (mostly obtained in powder form). TEM and HR-TEM demonstrate that the gadolinium-doped cobalt

ferrite nanoparticles are free spherical shaped crystal having a standardized allotment and no agglomeration.

6.3.2.2.4 Sol-Gel Method

In the case of metal oxide-based catalytic systems, a sol-gel method is the most promising process that has been proven by numerous studies over a decade. Likewise, present studies of sol-gel chemistry enlighten by giving several attractive strategies with techniques for material synthesis obtained from solution promoters. Through this process, the acquired catalytic system contains an exceptionally uniform arrangement of pore structure, low-temperature chemistry, reproducibility, high surface to volume ratio as well as component similarity.

A range of captivating sol-gel methods has been evolved by monitoring the operating conditions as well as modifying the physical and chemical properties of resources with the emersion of fresh subatomic chelating agents, and promoters with templates (Esposito 2019). $NiFe_2O_4$ nanoparticles are synthesized through this method and the attributes like single phase carrying an opposite spinel configuration, 9 nm grain size. The study reflects that the specific magnetic strength for the nanoparticle in powder form is much less than that of coarse-grained powders which proves that the sol-gel method is able to show the effects of grain size on the pore structure and magnetic properties as well as provide uniform high specific surface area magnetic catalyst at a low temperature (George et al. 2006).

6.3.2.2.5 Pyrolysis

One of the most familiar processes is pyrolysis through which synthesis of magnetic metal nanoparticles is possible. At a higher temperature, applying the generated carbon decreases the equivalent metal ions and helps to form additional active sites at Ni and Co nanoparticles. Apart from Ni and Co nanoparticles, his process is also applicable for the synthesis of carbon-based biomaterials like carbon solid base (Na_2SiO_3@Ni/JRC) catalyst originated from Jatropha curcas shell. Though the process is stable, it has a few disadvantages like easy agglomeration and also high energy consumption (Zhang et al. 2017).

6.3.2.2.6 Grafting

The grafting method is the most essential process only encountered for the synthesis of good quality magnetic organic-inorganic hybrid materials. Though the simple coating method is another method to get organic-inorganic hybrid materials, the configuration is not firm, loose, moreover easy to fall off. The grafting method is the only key to solving such a problem with the help of a covalent bond, it merges the active sites with the carrier. Not only that the covalent bond has ability to conform to

higher activity and outstanding recyclability of the catalyst. Interestingly, grafting the ionic liquid over the exterior surface of the amino group of Fe_3O_4 at NH_2-MIL-88b (Fe) MOFs offers a highly efficient well-organized magnetic ionic liquid catalyst, providing an amazing reusable quality (Wu et al. 2016). The characterization outcome explores the effectuality of grafting over the exterior surface of the amino group. An exemplary immobilized enzyme was synthesized with the help of silica-coated superparamagnetic iron oxide nanoparticles grafted in presence of an aldehyde group immobilized over the exterior surface of lipase (Karimi 2016).

6.3.2.2.7 Cross-Linking

The cross-linking method means coordination through coating or grafting which forms a network structure that provides a shield to the dynamic position and improves its constancy as well as an indissoluble collective configuration provided by the coupling reaction within the dual functional or multi effective reagent in addition to the amino group or carboxyl group in the enzyme particle where carboxyl group is essential after activation. For example, with the help of a cross-linker like glutaraldehyde acts on free lipase to provide immobilized lipase with magnetic nanoparticles, i.e., Fe_3O_4 at SiO_2 (Karimi 2016). Additionally, carriers like magnetic chitosan are mostly preferred for this process. Present studies reflect that the cross-linking method is a feasible as well as renowned synthesis process that offers an appropriate magnetic catalyst.

6.3.2.3 Magnetic Nanoparticle Synthesis

6.3.2.3.1 Preparation of Fe_3O_4 Nanoparticles

Being a well-known representative of the ferromagnetic family because of such excellence as high efficiency, configurable particle dimension, and well-built magnetism with fine dispersion. Fe_3O_4 nanoparticle frequently acts as a magnetic core that is obtained by coprecipitation, hydrothermal as well as reduction techniques. At a stable pH situation, initially precipitation through alkali (NH_4OH or NaOH); afterward, coprecipitation leads by dissolved divalent with trivalent iron salts within the distilled water. The well-known support synthesis process in recent studies is a solvothermal process employed to acquire Fe_3O_4 due to significant efficiency in controllable morphology along with particle dispersion of Fe_3O_4. During this course of action, $FeCl_3$ with trisodium citrate is diffused within ethylene glycol and NaCl. To obtain a larger particle of $FeCl_3$, initially, 30 min vigorous stirring helps the blend for proper mixing then stored into a stainless steel autoclave lined by applying PTFE then secured tightly which is kept at high-pressure sterilizer to maintain it at room temperature and cleanses using ethanol with deionized water repeatedly followed by the thermal treatment at conditions of 200 °C for 10 h. However, a number of studies confirmed that the more constructive effect acquired by the scattering technique is

the reduction of Fe_2O_3 into Fe_3O_4. In a classical procedure, are prepared, followed by to give (Tai et al. 2017). To get uniform as well as dispersed Fe_3O_4 nanoparticles; at first, Fe_3O_4 is obtained by H_2 reduction, afterwards, Fe_2O_3 is formulated by maturating of the $FeCl_3$ solution.

6.3.2.3.2 Preparation of γ-Fe_2O_3 Nanoparticles

Generally, γ- Fe_2O_3 nanoparticle is acquired by the sol-gel method (Ebrahimi et al. 2014). To form a clear solution, this process is carried out by mixing citric acid at a certain amount and dissolving $Fe(NO_3)_3{\cdot}9H_2O$ with KNO_3 blended into distilled water plus keeping the system at 100 °C till all salts are dissolved properly, And as the system temperature expands over 100 °C, in due course distilled water deactivates afterward gel formulation begins. Continuous heating of gel at 150 °C for 10 h leads to get required powder form afterward if acquired powder calcined by increasing temperature for a short time span as at 500 °C for 2 h helps to obtain the desired nanoparticles. Also, employing a simple coprecipitation method for Fe_3O_4 oxidation helps to get γ- Fe_2O_3 nanoparticles easily (Xie et al. 2017).

6.3.2.3.3 Preparation of Other Magnetic Nanoparticles

Advanced nanoscience has introduced numerous chemical methods to synthesize further metal-based (related to Ni and Co) magnetic nanocomposites like Ni^{-} SiO_2 magnetic nanocomposites (Fonseca et al. 2002).

For example, citric acid along with ethyl orthosilicate was diffused in ethanol, subsequently, nickel nitrate was dissolved and stirred well to get a homogeneous outcome. Later on, at the operating condition (e.g., 300 °C for 6 h) glycol was combined with the blend, followed by ball mill grinding, and ultimately at 500 °C thermal cracking to provide Ni-based magnetic nanocomposites. In presence of nitrogen flow, thermal cracking at a peak temperature (i.e., 900 °C) of ZIF 67 element carrying Co core helps to get magnetic Co-C composite elements that act as a classical ferromagnetic element, having qualities like well-built magnetic response-ability within the presence magnet, it has the ability to control hysteresis phenomenon accompanied by entire magnetic adsorption within 20 s.

6.4 Silica-Based Magnetic Nanoparticle Synthesis

6.4.1 Preparation of SiO_2-MX Support

Magnetite particles have a normal breadth of 561 nm with a standard deviation of 88 nm for $n = 139$ particles) were ready with the assistance of solvothermal technique and giving a thin silica shell cover utilizing Stober strategy to keep away from oxidation.

To set up the interaction utilizing the sol−gel strategy for getting the SiO_2-magnetic xerogel (SiO_2-MX support) was performed using tetraethyl orthosilicate (TEOS, Aldrich, 98%) as a silica source inside seeing the as-blended attractive particles. The standard weight degree was 65% attractive particles and 35% porous silica. A blend of HF/HCl liquid plan (1.8 $mol.L^{-1}$) was used as an impetus. Quickly, 1.5 g of alluring particles was dispersed in ethanol (7.0 mL) in presence of an ultrasonic shower for 30 min. Then, TEOS (3.0 mL) was added, and the mix was held under blending in with a glass mixing bar. After 30 min, the impetus game plan was added (2.0 mL), then, at that point gelation happens gradually. Dissolvable dispersal occurred under encompassing conditions longer than 7 days, and the powder arrangement was gradually gotten which is then washed with ethanol followed by refined water inside nonpartisan pH and subsequently vacuum dried for 2 h. The material was assigned SiO_2-MX.

6.4.2 Preparation of the Hydrophobic SiO_2/HPB-MX Support

The uniting strategy was used to merge the ionic silsesquioxane (IS) containing the decidedly charged 1,4-diazoniabicyclo-octane bunch with chloride as a counterion (Corma et al. 2008). For the joining over SiO_2-MX, an IS arrangement (25 mL, 20 g. L^{-1}) was added to 1.0 g of the material and put under mechanical mixing for 6 h. The strong stage was then attractively recovered and vacuum dried for 2 h. Following that, the material was through and through washed with water and a short time later ethanol and vacuum dried again and from that point on called SiO_2/IS-MX.

The hydrophobic moiety (stearate anion) was combined by particle trade. For this, 0.6 g of the readymade material with exchangeable chloride particles (SiO_2/IS-MX) was added to a sodium stearate watery arrangement (35 mL, 0.114 $mol.L^{-1}$, got from a stearic destructive response with NaOH) at an oil shower warming at 70 °C and under mechanical mixing. The structure was held under these conditions for 20 h. Hence, the strong was washed with warmed water (70 °C) and vacuum dried at 40 °C. The last material was relegated as SiO_2/HPB-MX.

6.5 Zeolite as a Support

In general, zeolites are broadly appointed solid catalysts almost in every chemical industry like crude oil catalytic cracking, Friedel–Crafts reactions, acid-catalyzed chemical transformations, and also in recent times biomass transformation (Xu et al. 2016). Zeolite enhances the gasoline yield that is about 30% because of this reason it is the most favorable catalyst for the oil refining as well as petrochemical industry (Abate et al. 2016).

The natural zeolites suffers different issues in terms of properties and applicability and hence there is a need to modify A, B, and X type of zeolites (Linde division of

Union Carbide, 1940) (Perego et al. 2017). The setups like translucent microporous aluminosilicates, with pore, estimates typically in the scope of 0.3–2 nm and having the uncommon capacity of zeolites fit as a fiddle particular catalysis which makes the zeolite extraordinary as perceived by logical society. They typically hold three-dimensional organizations of MO_4 tetrahedral units (M = Al, Si), connected through corner-shared oxygen molecules to develop exceptionally efficient interlinked pores. The permeable arrangement of zeolites can settle various cations, such as H^+, Na^+, K^+, and Mg^{2+}, just as sufficient if water particles.

Hence, observational recipe to clarify zeolites can be outlined by $M^{2/n}O\_Al_2O_3\_xSiO_2\_yH_2O$, where M = heterocation, n = cation valence, x = 2–200, and y = number of water atoms. The subbed cations are chiefly situated in apparently enormous pits built by adversely charged $[Si1\_xAlxO_2]x\_$ system, where x = 0.5 (Lowenstein's standard) (Busca 2017). In 2013, the expense of zeolite in the worldwide market was about 1.30 billion USD and it around increments to 1.76 billion USD in 2020, exhibiting the relentless extension of the zeolites industry.

Scarcely any unmistakable traits of zeolite like permeable design, shape selectivity, and solid corrosiveness help to partake in biomass valorization as an impetus (Ferrini et al. 2017). Particularly, the Bronsted/Lewis sharpness is a crucial property of zeolites that can strikingly close its reactant action and selectivity in biomass valorization (Hernando et al. 2018). The substitution of Si^{4+} by Al^{3+} in the tetrahedral structure inside zeolite is the way to acquire Bronsted corrosive destinations. The explanation is joining of Al^{3+} into the zeolite structure which makes negatively charged particles, that just can be overseen by cations to support the charge dependability (Querol et al. 2002). In the event that the proton (H^+) as subbed cation causes the solid Bronsted acridity inside zeolite considerably. Consequently, plainly it is noticeable that the expanding of Al/Si proportion (or abatement of Si/Al proportion) inspires the measure of Bronsted corrosive locales in the zeolite structure if in the event of protons involve all the cation destinations. Also, metals (like Ti, Zr, Hf, Nb, or Ta) incorporated into the zeolite design can fill in as Lewis acidic destinations. The upgraded hydrophobic conduct is an extra benefit given by metallozeolites, in light of this element its catalytical applications act in aqueous biomass transformations.

6.5.1 Cross-Linking Immobilization

The cross-associating immobilization measure joins the impetuses to one another using a multifunctional reagent (Ahmad and Sardar 2015). This system needn't bother with an assistance structure, and the resulting protein keeps up 100% activity (Sheldon 2007). In any case, an insufficiency of synthetic activity through conformational change can occur during immobilization. The control of the cross-associating reaction is inconvenient; thusly, it's hard to get a compound with high development support (Ahmad and Sardar 2015). Sheldon (2007) cultivated a cross-linked enzyme aggregates (CLEAs) system for convincing protein immobilization. The CLEAs system is especially direct and incorporates impetus precipitation from

watery courses of action by the extension of non-ionic polymers, salts, or normal solvents. The hydrolytic activities of the CLEAs and the lipases were worked on triple and twofold, independently, over those of free proteins.

6.5.2 Adsorption Immobilization

The adsorption of compound outwardly of assistance is an old development and a direct method. It relies upon a genuine limiting part, for instance, a dipole–dipole, hydrophobic, van der Waals cooperation, or hydrogen holding. Genuine limiting acted in modestly encompassing conditions and showed a high protein stacking. Adsorption immobilization doesn't give a high steadfastness and might cause a lack of impetus particles during action and washing by virtue of frail confining between the compound and the sponsorships. In the year 2004, Tang et al. immobilized glucose oxidase (GOD) on a platinum nanoparticle-changed carbon nanotube (CNT) cathode through adsorption. To avoid a lack of GOD, the outside of the GOD/Pt/CNT terminal was covered by Nafion. To beat the impetus sifting, the compound was immobilized into the pores of polyaniline nanofibers (PANFs) through a three-adventure measure, which included protein adsorption, precipitation, and cross-associating (EAPC) (Kim et al. 2013). In the year Kim et al. 2011, Kim et al. dissected GOD development and the strength of substance adsorption (EA), protein adsorption and cross-interfacing (EAC), and EAPC. The general activities of EA, EAC, and EAPC were 11%, 24%, and 100%, independently, and EAPC showed the most raised warm adequacy at 50 °C. The high unfaltering quality of EAPC can be explained by the extension in substance stacking and the evasion of compound sifting and denaturation.

6.5.3 Covalent Immobilization

The covalent immobilization of the biocatalyst is the association of the compound to the nanomatrix by covalent holding between the protein and the sponsorships. The strong limiting of the compound to the assistance network through the covalent bond thwarts impetus depleting from the surface and further develops the warm robustness some of the time (Hong et al. 2007). This technique, regardless, now and again prompts the deactivation of impetus considering the conformational constraint of the protein by covalent limiting (Kim et al. 2005). Dynamic limits (Km and Vmax) for nearby and immobilized compounds are moreover imperative to be settled. On this occasion, the Vmax worth of the immobilized compound is more humble than that of the neighborhood impetus. This shows that the substrate proclivity of the immobilized synthetic lessened in assessment with the nearby compound, which might be related to a steric effect and scattering requirement due to immobilization onto the sponsorships. Kim et al. encouraged an impetus all-out covering procedure on nanofibers. The basic activity of the synthetic absolute extended on various

occasions stood out from the example of simply a covalent immobilization protein, considering the way that the compound covering contained various layers of impetuses on the nanofibers. Concerning synthetic unfaltering quality, it is consistent without loss of everything except a month. This advancement can be applied to various nanomaterials and has likely applications in bioconversion, bioremediation, and biosensors (Liu et al. 2007).

6.5.4 Entrapment Immobilization

The entanglement innovation ensnares the compound in a penetrable gel or fibers. From a TEM examination, the distance across entrapped magnetite crystallites was around 20 nm. The catch collaboration can get protein development considering the unusual contact with the bound environment, which restricts the effects of gas bubbles, mechanical sheer, and hydrophobic solvents. Entrapment immobilizations using nanoparticles are all-around subject to the contrary micelle or sol-gel strategy. In the year 2013, Reetz et al. declared the simultaneous ensnarement of a lipase Amano PS (from *Pseudomonas cepacia*) and nanostructured magnetite (Fe_3O_4) containing hydrophobic sol-gel material. The colloidal magnetite-containing lipase was portrayed by compound development and was 2–3 times higher than that of free impetus. Yang et al. declared the simultaneous ensnarement of horseradish peroxidase (HPR) and round silica-shrouded nano-magnetite. This procedure contained two systems that acted in two phases: pivot micelle and sol-gel measures. Entrapment immobilization by rearranging micelle microemulsion can deliver uniform-sized nanoparticles, which prompts a strong mono dispersion of nanoparticles. Appeared differently in relation to free HPR, the nanoentrapment-immobilized HPR showed high trustworthiness toward temperature and pH changes. Nevertheless, this methodology required an intensive upgrade measure since it was difficult to control the contrary micelle size. Besides, the sol-gel measure included unforgiving reaction conditions for the snare immobilization. The new ensnarement advancement for proteins and nanoparticles using biomagnetic silica was performed under delicate conditions with an improvement in compound strength, immobilization viability, and stacking thickness. The single-compound nanoparticles (SENs) procedure was made by Kim and Grate in 2003. Each compound molecule was surrounded by a porous composite regular/inorganic association with a thickness of two or three nanometers.

6.6 TLL Immobilization on Magnetic Support

A TLL suspension was ready by the 125-overlap weakening of the TLL fluid plan, bought from Novozymes (Lipozyme TL 100 L), in phosphate cushion arrangement (PBS, 50 $mmol.L^{-1}$, pH 7.5, ionic strength of 0.12 $mol.L^{-1}$). Neutral pH was picked for immobilization for what it's worth in the ideal pH range for TLL action (7–10), a long way from the TLL isoelectric point (4.4), and this condition is likewise

announced for noncovalent immobilization of lipases (Lima et al. 2015). Then, 1200 μL of the as-prepared TLL suspension (0.079 U mL^{-1}) was offered to 20.0 mg of each help, SiO_2-MX or SiO_2/HPB-MX (the catalyst amount was advanced with respect to helping immersion tests information). The suspension stayed in touch with the supports under delicate shaking for 12 h at room temperature; thereafter, the backings were attractively recuperated and washed multiple times with PBS (50 $mmol.L^{-1}$, pH 7.5, ionic strength of 0.12 $mol.L^{-1}$). Supernatants and washing divisions were gathered for enzymatic action tests. The examinations were done in three steps. They are utilized in a wide range of responses, even mechanical, like hydrolysis, esterification, transesterification, and resolution of racemic blends, among others (Rios et al. 2018).

Most of the well-known lipases reported in the literature have their attributes like their dynamic sites, made out of serine, histidine, and aspartic corrosive/glutamic acid (Kim et al. 1997), is blocked by a polypeptide chain called a lid, which opens within the sight of a hydrophobic interface (Verger 1997). In its open (dynamic) configuration, lipase is interfacially activated (Schmid and Verger 1998). The actuation of lipases on hydrophobic interfaces has been utilized as an effective system to immobilize lipases in their open conformity on the outside of hydrophobic supports. Lipase from *Thermomyces lanuginosus* (TLL) is one of the principal lipases utilized in the fine chemicals industry, biodiesel production, and food industry (De Lima et al. 2018). Its structure was settled in a spherical format and a size of 3.5 × 4.5 × 5.0 nm (Fernandez-Lafuente 2010). Along these lines, TLL is a brilliant possibility for immobilization in upholds containing pores inside the scope of mesopores (2–50 nm).

In this work, magnetic silica was ready through condensation reactions of silica forerunners within the sight of magnetite. The amalgamation was planned so the material's pore size would be viable with TLL catalyst measurement. Anionic silsesquioxane was united on its surface; later, its counterion was particle traded with stearate to give hydrophobicity. This mesoporous, hydrophobic, and magnetic material was utilized as a help for the TLL chemical and further assessed as a recyclable biocatalyst for the model response p-nitrophenyl palmitate hydrolysis.

6.7 Co-immobilization

Various researchers have deliberated on the benefits to employ enzyme immobilization that liberated outcome obtained by a primary afterward short interval of diffusion assists in transmission to the subsequent co-immobilized one. To attach triacylglycerols ester, this particular technique is appropriate where the acyl-migration step may easily get accessible by such comprehensive dynamicity obtained by blends of non-specific lipase with 1,3-specific lipase. In this case, lipases (named *Rhizopus oryzae* lipase and *Candida rugosa* lipase kept with silica gel) are generally simultaneously take part with *Thermomyces lanuginosus* lipase to attack *Pichia pastoris* cell's exterior periphery (Lee et al. 2013; Ji et al. 2016), and

Rhizomucor miehei in addition to *Candida antarctica* lipases on epoxy-functionalized silica (Shahedi et al. 2019; Babaki et al. 2017).

Lipases are co-immobilized on the same substance periphery with the aim of enhanced comprehensive dynamicity and better enzyme explicitness and enantioselectivity for hydrolysis of triacylglycerols on top of those acquired di-acylglycerols along with monoacylglycerols that have to be furthermore hydrolyzed (Yan et al. 2011). Howsoever, the most active enzyme may get diminished activity during this process (Arana-Peña et al. 2020). Diversification of the co-immobilization approach was projected by numerous lipases immobilized in layers also termed polyethyleneimine coating (Virgen-Ortíz et al. 2017). Several scientists have applied PEI/glutaraldehyde coating over *Candida antarctica* that is obtained from *Thermomyces lanuginosus*, *Rhizomucor miehei* along and phospholipase Lecitase Ultra blend provides five enzyme layers both for lipases A and B (Arana-Peña et al. 2020). While it provides a modern approach for fats hydrolysis, several inconveniences may occur due to high lipase expenditure, steric hindrance, or inhibition overactive location caused by applied coating agents, triacylglycerols spare accessibility.

6.8 Conclusion

This manuscript provides a compact effort to present concepts like enzyme implementation on top of other catalysts, expanded scope of enzymatic immobilization, and enzyme entrapment using nano-support. Numerous specialists have successfully immobilized lipases on functionalized nanomaterials, and their applications seem promising. Specifically, the utilization of nano-immobilized lipase in pressed bed reactors brought about high catalyst stacking, numerous reuses, and successful insurance from chemical denaturation in biodiesel creation, showing the capability of nano-immobilization innovation in the biofuel business. Further examination, particularly for the scale-up of the biodiesel creation measure, utilizing nano-immobilized lipase is important to execute these advancements on a mechanical level. The incorporated improvement of a high chemical and nano-immobilization strategy will assume a critical part in practical biodiesel creation.

Competing Interests The authors declare that they have no competing interests.

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Chapter 7
Valorization of Dairy Industry Waste into Functional Foods Using Lactase

Tapas Palai and Kashyap Kumar Dubey

Abstract India, with a vibrant dairy industry, has the possibility to dominate the domestic and international market through the development of specialized functional foods. Whey, the liquid left behind after the manufacturing of cheese, is considered to be waste material and is of huge concern to the dairy industries for its disposal in the open environment. Whey contains a considerable amount of lactose (~6% w/v) apart from various proteins. Lactose can be converted to galacto-oligosaccharides (GOS) by the trans-galactosylation reaction catalyzed by lactase enzyme. This could solve two-fold objectives; firstly, the minimization of lactose in the dairy waste, and secondly, the value addition to the waste by producing GOS, a functional food/nutraceutical and prebiotic food ingredient which is beneficial for lactose-intolerant people, especially the babies and diabetic patients. Designer food products having several health benefits find the increasing acceptance in the prevention and treatment of disease. This chapter will explore the efficacy of lactase for the hydrolysis of whey to produce GOS. The main focus will be on the effective valorization of whey to produce valuable products using enzymatic methods, mechanistic pathways, enzyme origin, recent advances, and challenges for commercialization.

Keywords Whey · Lactose · Galacto-oligosaccharides · Lactase · Nutraceutical · Functional foods

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Abbreviations

BCAAs	Branched-chain amino acids
BOD	Biological oxygen demand
BSA	Bovine serum albumin
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
DP	Degree of polymerization
ED	Electrodialysis
FAO	Food and Agriculture Organization
FOS	Fructo-oligosaccharides
GMP	Glycomacropeptide
GOS	Galacto-oligosaccharides
IDF	International Dairy Federation
MF	Micro-filtration
MWCO	Molecular weight cut-off
NF	Nano-filtration
PBR	Packed bed reactor
PFR	Plug flow reactor
RO	Reverse osmosis
UF	Ultra-filtration
WPC	Whey protein concentrates
WPI	Whey protein isolates
XOS	Xylo-oligosaccharides

7.1 Introduction

In modern civilization, milk and fermented dairy products such as curd, cheese, casein, buttermilk, yogurt, etc. are an integral part of daily diet and are essential for the growth of human beings. Dairy products contain crucial ingredients for a balanced diet (Gasmalla et al. 2017). In addition, they also provide health benefits such as blood pressure control, prevention of obesity, osteoporosis, dental caries, cardiovascular diseases, hypertension, colorectal cancer, bone ailments, aging, etc. (Nagpal et al. 2012). Due to the steep increase in consumption of fermented milk products, the dairy industry witnessed huge growth during the last few decades. About six billion people worldwide (approximately 80% of the total population) regularly consume liquid milk or other dairy products.

Food and Agriculture Organization (FAO) of the United Nations reported that the global milk and milk products production reached 906 million tonnes in 2020, which is 2% higher than the previous year. International Dairy Federation (IDF) in its report claimed that the global cheese production was increased by 13% and 16% in 2017 and 2018, respectively (Refer: IDF report 2017–2018). The production of whey is

directly related to the production of cheese and other dairy products. In general, approximately 9 L of liquid whey are obtained from the production of 1 kg of cheese. The global whey production was estimated at 200 million tonnes per annum in 2016 (Lappa et al. 2019) which is expected to increase every year.

Whey contains several milk proteins, lactose, and minerals. Being a waste having rich organic content, it has a very high level of Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD). Hence, it is a huge challenge for the dairy industries across the globe to discharge such a large quantity of whey into open streams. One possible way could be the valorization of whey to produce value-added products such as milk proteins, lactose, organic acids, and functional foods (Kaur et al. 2020).

During the last three decades, there has been a growing interest in functional foods especially prebiotic and probiotic food ingredients. The prebiotic food ingredients can augment health and/or enhance disease resistance when consumed in the right quantity. Galacto-oligosaccharides (GOS) are one such prebiotic food that can offer significant therapeutic effects to the human body. GOS can be synthesized from lactose or whey by the enzymatic action of lactase. This will reduce the organic load of the whey stream and can be maintained the discharge within the permissible limits of respective components.

This chapter will provide an insight into comprehensive information about whey and its characteristics, separation of various value-added products, valorization of whey and lactose to GOS through the enzymatic method, and an overview of the enzyme sources and mechanism, recent development, and prospects.

7.2 Whey

Whey is the liquid left behind after the production of cheese, casein, yogurt, etc. from milk. It constitutes approximately 80–90% (v/v) of milk and contains about 50% of the nutrients (protein, lactose, vitamins, and minerals) in the original milk (Teknotext 1995). It was discovered in ancient times (before 3000 years). However, it was then considered as either a waste material or as animal feed. With advanced technologies, whey can be converted into value-added products. The global market of whey powder and whey proteins was approximately estimated at USD 6.8 billion in 2019 and is forecasted to reach USD 7.8 billion by 2024 (Refer: Wheybook-2020, International-Dairy.com). It is considered one of the biggest reservoirs of food proteins and lactose, a precursor material for GOS synthesis.

7.2.1 Classification of Whey

Whey is generated by the coagulation of milk. Different methods of coagulation yield different types of whey. Cheese whey (also known as sweet whey) is produced

Table 7.1 Compositions of various types of whey (de Wit 2001; Papademas and Kotsaki 2020; Teknotext 1995)

Constituents/Parameters	Cheese whey/Sweet whey	Acid whey/Sour whey
pH	6.0–7.0	4.0–5.0
Total solids (g/L)	63.0–70.0	63.0–70.0
Water (g/L)	930.0–927.0	930.0–927.0
Fat (g/L)	0.2–0.5	0.3
Protein (g/L)	6.5	6.1
Non-protein nitrogen (NPN) (g/L)	1.8	1.8
Lactose (g/L)	46.0–52.0	44.0–47.0
Mineral ash (g/L)	5.0	8.0
Calcium (g/L)	0.4–0.6	1.2–1.6
Phosphorous (g/L)	1.0–3.0	2.0–4.5
Sodium (g/L)	0.5	0.5
Potassium (g/L)	1.6	1.6
Chloride (g/L)	1.1	1.1
Lactic acid (g/L)	2.0	6.4

as a by-product of cheese production. Acid whey (also known as sour whey) is produced from the casein production that is precipitated by mineral acids. Cheese whey (accounts for approximately 85% of total whey) and acid whey (accounts for approximately 15%) are the most common types. A slight change in the composition may be observed for various types of whey obtained from different processes such as Gouda whey (generated from the cheese production with the help of *Lactococcus lactis*) and Camembert whey (generated from the production of Camembert cheese). Liquid cheese contains approximately 93.0% water and 7% total solids. Lactose is the major constituent in the total solids. The typical composition and physical characteristics of various types of whey are given in Table 7.1.

7.2.2 Applications

In the 1970s, whey was used in whey baths owing to its beneficial active ingredients having anti-inflammatory qualities and skin nurture properties. However, such uses kept on decreasing in the nineteenth century because of environmental issues associated with whey. It was considered either waste or used for feeding the animals (Papademas and Kotsaki 2020). Whey contains a considerable amount of lactose, health-promoting proteins, and other minerals as mentioned earlier (Table 7.1). With the development of process technology, either whey or its ingredients finds various important applications in food, pharmaceuticals, dairy, and beverage sectors and may replace skim milk powders in the formulation. Figure 7.1 summarizes some of the important applications. However, for specific applications, it is required to separate the specific components from whey. Whey needs to be demineralized to

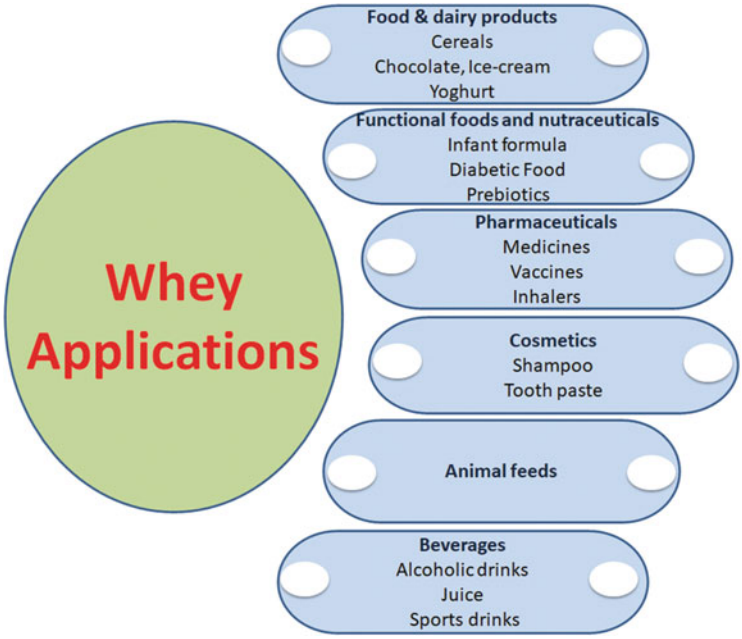

Fig. 7.1 Important applications of whey

varying degrees for use in infant formulas which requires finely controlled compositions of minerals. Whey protein concentrates (WPC) or whey protein isolates (WPI) may be obtained by concentrating the protein present in whey by membrane technology. Immune-stimulating protein lactoferrin, lactoperoxidase, and bovine serum albumin (BSA) can be isolated and purified using a combination of membrane filtration and chromatography techniques. These proteins find their applications in high-protein products. Lactose separated from whey may be used in infant milk formulas, as a carrier in pharmaceutical tablets, as standard for milk powders, baked food, confectionery, dairy, cosmetics, beverages, etc. (Rocha and Guerra 2020).

7.2.3 Environmental Impact

A huge quantity of wastewater is generated from the dairy industry. It has been reported that the volume of wastewater is approximately 2.5 times higher than that of the volume of processed milk (Slavov 2017; Goswami et al. 2020). Whey, a major waste generated from the dairy industry, will cause environmental pollution if it is discharged into the open streams without any treatment. Owing to the high content of organics (lactose, slowly degradable proteins, and lipids), the waste stream has a very high value of BOD and COD. BOD is defined as the amount of oxygen consumed by aerobic microorganisms for the decomposition of organic material

present in a water sample at a certain temperature over a specific period. The BOD value is, in general, expressed in mg of oxygen consumed per L of the sample at 20 °C during 5 days (BOD_5) or 7 days (BOD_7) or ultimate 20 days (BOD_u) period. On the other hand, the COD indicates the amount of oxygen required to oxidize the pollutants in wastewater using a chemical oxidant. The ratio (BOD/COD) indicates the ease of biodegradability. The higher the values, the better will be the biodegradation property (Teknotext 1995). For dairy wastewater, a typical value of this ratio varies between 0.4 and 0.8. The highest COD and BOD_5 values of whey are reported to be between 60–80 and 30–50 g/L, respectively (Slavov 2017) which is much higher than the permissible limit. Hence, either removal of toxic components from dairy wastewater or valorization of whey is very essential.

7.3 Valorization of Whey

Whey valorization may solve two-fold objectives.

(a) It decreases environmental pollution by separating the various components from whey.
(b) Several value-added products may be produced from the whey which is beneficial for inpatients, infants, and lactose-intolerant people.

Only removal of the organic components will decrease the BOD, COD, and total solids from the wastewater. But valorization may add value to the waste by converting them into useful products. Lactose and milk proteins present in whey may be directly used for various purposes after separation and purification using different physiochemical (precipitation, flocculation, etc.) and separation techniques (ultrafiltration (UF), microfiltration (MF), nanofiltration (NF), reverse osmosis (RO), extraction, etc.). Furthermore, the whey components may be converted to valuable products using chemical and biochemical techniques. The latter is considered to be an environmentally friendly method (Kaur et al. 2020). Figure 7.2 depicts the outline of various processes involved in the valorization of whey. Some of the commercially available products from whey and their synthesis techniques are discussed in the subsequent section.

7.3.1 Whey Powder

Whey powder may be obtained from fresh whey solution by pasteurization followed by drying in a drum or spray dryer. Pasteurization is carried out to reduce bacterial activity and to increase the shelf life. It contains all the ingredients of fresh whey. Demineralized whey can be obtained by partially removing the minerals by ion exchange, diafiltration, and electrodialysis (ED) methods (Guo and Wang 2019). Spray drying offers the advantage of getting whey in the powdered form directly,

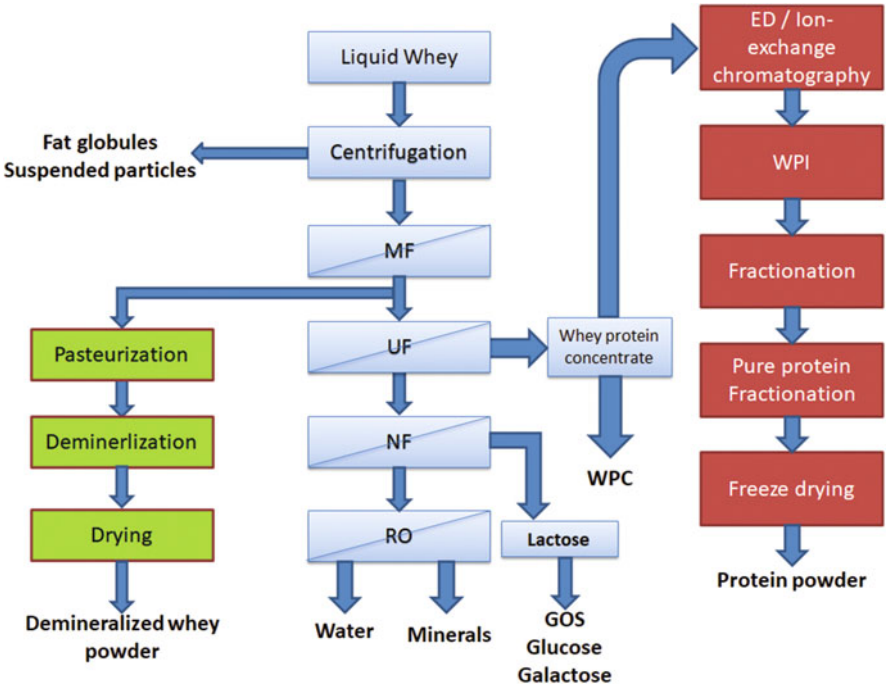

Fig. 7.2 Outline for whey valorization process

and the finished product obtained has a non-hygroscopic property that prevents agglomeration. Whereas the drum dryer produces a layer of whey that needs to be scraped off and the powder remains in agglomerated form. The latter technique is not in use nowadays (Teknotext 1995). Whey powder finds its application as an ingredient in several products such as calf milk replacers, infant formulae, beverages, bakery and confectionery, ice cream, and other dairy products. In general, whey powder offers a low-cost source of protein, carbohydrates, and minerals although it has little contribution to product functionality (Zadow 2003).

7.3.2 Whey Proteins

Whey is an abundant source of low-cost proteins. It contains about 11% proteins in the total solids present in whey. Proteins are long-chain, large molecular weight complex molecules that are made of a large number of amino acids and play many critical roles in the metabolic system. It consists of several proteins such as β-Lactoglobulin (β-Lg), α-Lactalbumin (α-La), Immunoglobulins (IGs), BSA, Lactoferrin, Lactoperoxidase, branched-chain amino acids (BCAAs),

Glycomacropeptide (GMP), etc. Some of the physicochemical properties and their biological activities are shown in Table 7.2.

The proteins can be separated from the whey liquid in a series of separation and purification techniques. Centrifugation of whey liquid at 11000 rpm may partially remove the fat and suspended particles. Microfiltration (MF) of the supernatant employing 0.45 μm pore size membrane can separate the remaining suspended casein and fat particles. The molecular weight cut-off (MWCO) of most of the proteins present in the whey is higher than 8.0 kDa. Hence, UF of the permeate solution with 7–8 kDa MWCO membrane will lead to the separation of whey proteins as retentate (Das et al. 2016). Thus, the whey protein can be concentrated up to 80% of total solids, known as WPC. Further purification with ion-exchange chromatography will produce up to 90% protein solution, known as WPI. Further fractionation of various proteins can be done by applying chromatographic methods (ion-exchange, adsorption, or affinity chromatography) followed by selective elution or membrane separation (e.g., ED) or a combination of both. The conventional protein fractionation techniques (salting out, iso-electric precipitation, etc.) are not useful for large-scale operations (El-Sayed and Chase 2011). The isolated proteins may be freeze-dried under a vacuum to obtain in powdered form.

7.3.3 Minerals

Whey contains several abundant elements such as calcium, sodium, potassium, magnesium, and phosphorus in major amounts and zinc, iron, copper, and manganese in minor amounts. The minerals can be obtained from the permeate of the lactose separation (refer to Sect. 7.3.4) by utilizing the RO method or by drying the permeate (Guo and Wang 2019; Rocha and Guerra 2020).

7.3.4 Lactose

Lactose or milk sugar is a disaccharide (galactose unit followed by glucose) having the chemical formula β-D-galactopyranosyl-(1 → 4)-D-glucose. It can be separated from whey by two methods:

(a) Crystallization: Lactose can be crystallized from the concentrated whey solution. It depends on several factors such as degree of supersaturation, the availability of crystal surface for nucleation and growth, purity of the solution, temperature, viscosity, agitation of the crystals in the solution, etc. Crystallization yields a pure form of lactose crystals which can be further separated from the mother liquor by centrifugation or filtration (Teknotext 1995).
(b) Membrane separation: Various membrane separation techniques can separate lactose from dairy waste. The permeate from the UF unit (from the protein

Table 7.2 Composition, molecular weight, and biological activities of whey proteins (Papademas and Kotsaki 2020; Ramos et al. 2016)

Whey protein	Composition in whey (% of total proteins)	Molecular weight (kDa)	Biological activities
β-Lactoglobulin	50.0–58.0	18.3	• Act as a transporter of retinol, palmitate, vitamin D, cholesterol, fatty acids, and triglycerides • Regulate mammary gland metabolism • Show anticarcinogenic, immunomodulatory, antihypertensive, antioxidant, antithrombotic, and antimicrobial properties • Reduce stress by increasing brain serotonin levels • Improve liver function • Reduce blood pressure
α-Lactalbumin	11.0–20.0	14.2	• Have anticarcinogenic activity • Application in lactose synthesis • Treatment of chronic stress-induced disease • Helps in the absorption of minerals • Possesses antibacterial, immunomodulatory, and antitumor activities
Immunoglobulins	10	150–900	• Show immunomodulatory, antimicrobial, opioid activities • Disease protection in the newborn through passive immunity • HIV treatment
Bovine serum albumin	6.0–10.0	66.4	• Act a principal carrier of fatty acids and other lipids • Possess antimutagenic, antioxidant, anticarcinogenic activities • Source of essential amino acids
Lactoferrin	1.0–3.0	80.0	• Show antimicrobial, antithrombotic, immunomodulatory, antiproliferative, antioxidant activities
Lactoperoxidase	0.25–0.5	78.5	• Antimicrobial, immunomodulatory activities
Branched-chain amino acid	–	–	• Have great importance to athletes as directly metabolized into the muscle tissue • The first amino acids used during periods of exercise and resistance training • Increase satiety, protect against muscle-protein loss, enhance muscle-protein synthesis, and improve glycemic control

(continued)

Table 7.2 (continued)

Whey protein	Composition in whey (% of total proteins)	Molecular weight (kDa)	Biological activities
Glycomacropeptide	10.0–15.0	8.0	• Act as amino acid sources for phenylketonuria patients • Protect against toxins, bacteria, and viruses, promote bifidobacterial growth, and modulate the immune system

separation) can be further subjected to Nanofiltration (NF) with a membrane of MWCO of 0.15–0.30 kDa to produce the retentate containing concentrated lactose which can be further crystallized or separated by ion-exchange or ED methods (Das et al. 2016).

Lactose is commonly used in infant formula, confectionery, bakery, dairy, and pharmaceutical products. However, food industries use a very small fraction of total lactose. Furthermore, a limited quantity of lactose is to be used owing to several properties such as low solubility; susceptibility to crystallization in aqueous solution at low temperatures; lower calorific value; reduced sweetness; and lactose intolerance in infants and diabetic patients (Palai 2015). Hence, lactose can be converted to other value-added products that are summarized in the next section.

7.3.4.1 Lactobionic Acid

Lactobionic acid (4-O-β-galactopyranosyl-D-gluconic acid) can be synthesized by catalytic oxidation of lactose. The free aldehyde group present in the lactose is converted to carboxylic acid. It can also be synthesized by microbial method (using *Pseudomonas* species) or enzymatic method (using cellobiose dehydrogenase). It has anticoagulant, antithrombotic, antioxidant, and wound healing properties. It is most widely used in the cosmetic, food, and dairy industries (Rocha and Guerra 2020).

7.3.4.2 Lactitol

Lactitol [4-(β-D-galactopyranosyl)-D-sorbitol] is a sugar alcohol and may be obtained from lactose by hydrogenation with tetrahydoborate. It is a non-carcinogenic sweetening agent having low calorific values and is used in jellies, toppings, and sugar-free foods for diabetic patients (Rocha and Guerra 2020).

7.3.4.3 Functional Foods

Various functional foods such as lactulose [4-O-β-galactopyranosyl-D-fructose] and GOS can be synthesized from lactose by enzymatic hydrolysis. These products have prebiotic activities. Details about functional foods are discussed in the next section.

7.4 Functional Foods

Functional foods are the food ingredients or supplements having health and disease-preventing benefits beyond their nutritional value. Japan is the first country to coin the term "functional food" in the early 1980s and to formulate a specific regulatory approval process in 1991. Functional foods, in general, converge with nutraceuticals, medical foods, prebiotics, designer foods, pharmafoods, vitafoods, etc. (Arihara 2014; Mehariya et al. 2021; Goswami et al. 2021, 2022). The conventional sources of functional foods include fruits, vegetables, herbal products, and beverages, whereas modified functional foods include fortified milk and dairy products such as fermented milk and yogurt, etc.

The term "nutraceutical" was coined by Stephen L. DeFelice in 1989. It consists of two words, "nutrition" and "pharmaceutical" (Kalra 2003). Nutraceuticals are food ingredients that have medicinal properties and have a physiological benefit or protect against chronic diseases apart from their normal nutritional value. Oligosaccharides such as GOS, fructo-oligosaccharides (FOS), xylo-oligosaccharides (XOS) show nutraceutical activity. On the other hand, prebiotics was discovered by Marcel Roberfroid (Roberfroid 2007) in 1995 and are defined as a non-digestible food ingredient having beneficial effects on the host body. It selectively stimulates the growth and/or activity of bifidobacteria present in the colon, thus improves the health of the host (Palai 2015). Some common prebiotics are inulin, lactulose, oligosaccharides such as GOS, FOS, XOS, etc. (Khandekar et al. 2014; Palai et al. 2012). Among all the oligosaccharides, the GOS is the most common and can be synthesized by the valorization of lactose present in whey.

7.5 Galactooligosaccharides

GOS is considered a functional food and a nutraceutical and is of great interest in the food and dairy industries. It is a non-digestible oligosaccharide and is a complex mixture of various sugars. It is a chain of galactosyl-galactose with a terminal glucose moiety. The general structure of GOS may be represented as $(\text{galactose})_n$-glucose (Czermak et al. 2004), where n is 2–9. Generally, the terminal galactose-glucose bond is of β-(1–4) type glycosidic linkage, which is the basis of categorizing GOS into various types such as β-(1–3), β-(1–4), and β-(1–6) (Fig. 7.3). The β-(1–4)

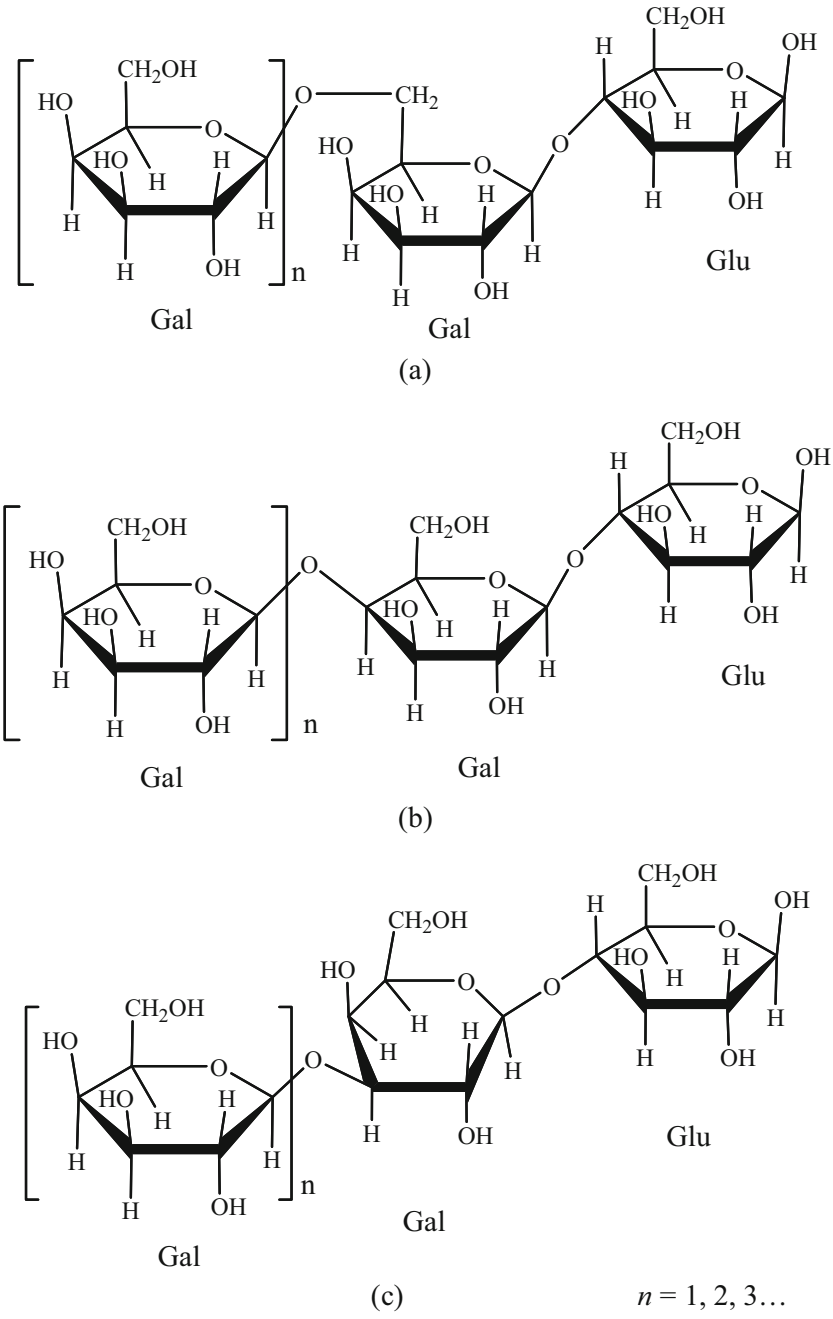

Fig. 7.3 General structure of various GOS; (**a**) β-(1–6), (**b**) β-(1–4), and (**c**) β-(1–3) type. *Gal:* galactose, *Glu:* glucose (Palai 2015)

and β-(1–6) are the most common among all. GOS finds its most significant application in infant formula and foods to be consumed by diabetic and obese patients. Apart from this, it is also used in fermented milk products, bread, jam, ice cream, and several beverages (Palai 2015).

7.5.1 Physicochemical Properties

GOS has high solubility and relatively less sweetness (about 35% that of sucrose). The structure of oligosaccharides and degree of polymerization (DP) or chain length are responsible for lower sweetness (Mussatto and Mancilha 2007). They have a higher viscosity than corn syrups containing high fructose, lower water activity, lower freezing point, and relatively high moisture retention capacities. GOS is highly stable at high temperatures and over a variable pH range. They possess a relatively low caloric value (1.0–2.0 kcal/g) because of the difficulties that arise to hydrolyze and absorption in the upper gastrointestinal tract (Sako et al. 1999).

7.5.2 Health Benefits

GOS has several beneficial effects on human health. Owing to their β-configuration, human saliva and gastric enzymes (which are generally α-specific) cannot hydrolyze GOS (Mussatto and Mancilha 2007). Hence, after consumption, it reaches the colon without getting absorbed in the upper intestinal tract and gets fermented selectively by beneficial *Bifidobacterium* sp. to produce short-chain fatty acids (Lamsal 2012; Maischberger et al. 2008). In the lower intestine, GOS selectively stimulates the growth of *Bifidobacteria* that provides several beneficial activities such as retardation of potentially harmful bacteria such as *Clostridia* and *Bacteroides* species in the gut (Oku 1996). Apart from these, GOS also provides several other health benefits in humans and animals, such as stool improvement, better absorption of minerals, carcinogenesis, and allergy alleviation (Lamsal 2012). However, consumption of GOS in excess amounts should be avoided because of the osmotic effect that transfers water into the large intestine. Hence, it may lead to intestinal discomfort, flatulence, diarrhea, and other health issues (Roberfroid and Slavin 2000).

7.5.3 Production of GOS

7.5.3.1 Extraction from Natural Resources

Traces amount of GOS is present in milk and legumes plants such as lentils, chickpeas, beans, and certain root vegetables and can be extracted by applying the

solvent extraction technique. However, because of the lower quantity of GOS in those natural sources, the extraction process becomes very difficult and economically not feasible (Sako et al. 1999).

7.5.3.2 Chemical Synthesis

Oligosaccharides may be produced by hydrolysis of the precursor polysaccharides using mineral acids as catalysts (Ãvila-Fernandez et al. 2011). Hydrolysis of lactose can be performed using mineral acids catalysts. However, the process requires high acid concentrations and high temperatures. Furthermore, several by-products are also formed during acid-catalyzed hydrolysis that leads to the non-equimolar formations of glucose and galactose as compared to the amount of lactose converted (Hatzinikolaou et al. 2005). Hence, this method is generally less preferred for the synthesis of GOS.

7.5.3.3 Enzymatic Synthesis of GOS

The enzymatic method is, in general, preferred over the chemical synthesis as it offers high selectivity towards the substrate. The synthesis of GOS can be obtained by the treatment of lactose recovered from whey or fresh whey with β-galactosidase (also known as lactase) enzyme. Thus, whey can be valorized more effectively. Lactose hydrolysis catalyzed by lactase is found to be very complex. The enzyme catalyzes two simultaneous reactions: (a) hydrolysis reaction and (b) trans-galactosylation reaction.

7.5.3.3.1 Hydrolysis Reaction

In the hydrolysis reaction, the lactase enzyme converts lactose into glucose and galactose by hydrolyzing β-(1–4) glycosidic linkage (Fig. 7.4a). This is an undesired reaction during the formation of GOS.

7.5.3.3.2 Trans-Galactosylation Reaction

In trans-galactosylation reaction, on the other hand, the lactase converts lactose to tri-saccharides where lactose serves as a galactosyl acceptor (Fig. 7.4b) (Palai 2015). The tri-saccharides thus formed further act as a galactosyl acceptor to form tetra-saccharides. Subsequently, a series of similar trans-galactosylation reactions will result in a mixture of GOS of varying DP (Mahoney 1998).

In an aqueous solution, both trans-galactosylation and hydrolysis reactions compete with one another. The trans-galactosylation reaction predominates when lactose concentration in the solution is very high, i.e., at the early stage of the reaction or

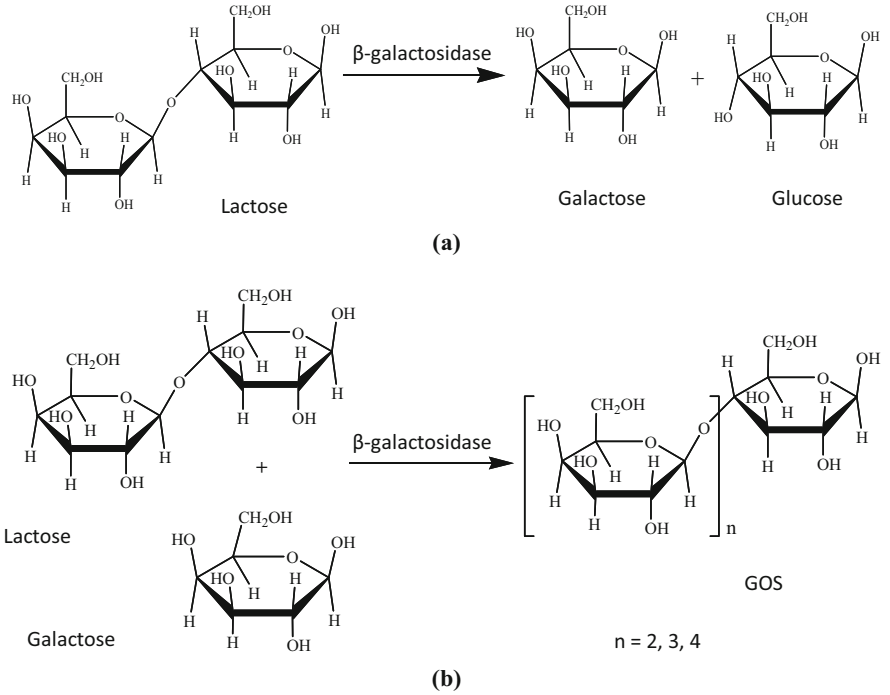

Fig. 7.4 Schematic representation of (**a**) hydrolysis reaction and (**b**) trans-galactosylation reaction during lactose conversion using lactase enzyme (Palai 2015)

starting with a very high initial lactose concentration. Whereas hydrolysis reaction predominates at lower lactose concentration, i.e., at the later stage of the reaction or starting with low lactose concentration. As a result of the coexistence of both the reactions, the reaction mixture always contains a considerable amount of unreacted lactose apart from glucose, galactose, and GOS of various DP (Mahoney 1998; Prenosil et al. 1987a). The yield of GOS (on a dry basis) is defined as per Eq. (7.1).

$$\text{Yield}(\%) = \frac{\text{Mass of individual component}}{\text{Total mass of all saccharides}} \times 100 \tag{7.1}$$

The major drawback of lactase catalyzed synthesis of GOS is that the equilibrium always favors the hydrolysis over trans-galactosylation reaction in an aqueous solution, which tends to decrease the yield of GOS (Petzelbauer et al. 2000). Hence, to maximize the GOS yield, a high concentration of lactose is to be maintained apart from other operating parameters like pH and temperature. However, there is a concern over the yield of GOS as the maximum yield was reported in most of the literature to be in the range of 40–41% (Akiyama et al. 2001). A study by Shin et al. reported a comparatively high yield of GOS using batch (yield 54%) and continuous (yield 55%) modes (Shin et al. 1998).

7.5.4 Origin of Lactase

The enzyme, β-galactosidase (EC 3.2.1.23) (also known as lactase) is well established and commonly utilized for the synthesis of GOS from lactose or whey. Lactase may be isolated from animals, plants, and several microorganisms (yeasts, fungi, bacteria, and archaea). The lactase that is derived from microbial sources plays an important role in the industrial process and is known to provide higher yields of GOS as compared to other sources (Grosova et al. 2008). The operating conditions for maximum activity of the enzyme and hence the GOS yield are also dependent on the source of the enzyme. Table 7.3 shows common microbial sources of lactase and the optimum conditions for GOS synthesis. The type of glycosidic linkage in GOS depends on the sources from which the enzymes are derived. Lactase derived from *Bacillus circulans* or *Cryptococcus laurentii* mainly produces β-(1–4) glycosidic linkages, whereas the enzymes derived from *Aspergillus oryzae* or *Streptococcus thermophilus* are known to produce predominantly β-(1–6) type of GOS (Sako et al. 1999). Lactase from *Bacillus circulans* also can produce a relatively higher amount of GOS with a higher DP (tetra- and penta-saccharides), whereas tri-saccharides formation occurs majorly utilizing enzymes from *Aspergillus oryzae* (Albayrak and Yang 2002; Iwasaki et al. 1996) and *Kluyveromyces* sp. Sources (Boon et al. 2000; Prenosil et al. 1987b).

Table 7.3 Common microbial sources of lactase and the optimum operating conditions for GOS synthesis

Microbial domain	Enzyme source	Optimum operating conditions	
		Temperature(°C)	pH
Yeast	*Kluyveromyces lactis*	30–45	6.0–7.0
	Kluyveromyces fragilis	30–45	6.0–6.5
	Bullera singularis	50	5.0
	Kluyveromyces lactis	40	7.0
Bacteria	*Escherichia coli*	35–40	6.5
	Bacillus circulans	40	6.0
	Lactobacillus reuteri	37	6.5
	Lactobacillus thermophilus	55	6.2
	Sterigmatomyces elviae	60	5.0
	Thermotoga maritima	80	6.0
Fungus	*Aspergillus aculeatus*	45	6.5
	Aspergillus niger	55	3.5–4.5
	Aspergillus oryzae	40	4.5
Archaea	*Pyrococcus furiosus*	80	5.0
	Sulfolobus solfataricus	80	6.0

7.5.5 Hydrolysis Mechanism

Very little information is known about the actual mechanism that occurs during the lactose conversion as it is very complex. Several authors have proposed kinetic models for GOS formation and validated them from experimental observations. Most of the studies are based on the Michaelis–Menten kinetics.

The Michaelis–Menten kinetics is a simple two-step reaction mechanism proposed by Leonor Michaelis and Maud Menten. The first step proposes the reversible binding of the substrate (S) with enzyme (E) to form an enzyme–substrate complex (ES). The ES complex formed irreversibly gets converted to product (P) in the subsequent step and releases the enzyme. The mechanism is presented in Eqs. (7.2)–(7.3) (Shuler and Kargi 2002).

$$E + S \rightleftarrows ES \tag{7.2}$$

$$ES \rightarrow E + P \tag{7.3}$$

GOS synthesis catalyzed by lactase involves hydrolysis and trans-galactosylation reactions. Furthermore, the enzyme activity and hence GOS synthesis may get inhibited by the presence of a high concentration of lactose (known as substrate inhibition) or the products such as glucose and galactose (competitive inhibition by the product). Most of the studies revealed competitive inhibition of the enzyme by glucose. In an earlier study (Palai et al. 2014b), a five-step reaction mechanism (Eqs. 7.4–7.8) was proposed and found to be in good agreement with experimental data for describing GOS formation from lactose. This model considers the competitive inhibition by glucose apart from the hydrolysis and trans-galactosylation reaction. However, the formations of all oligosaccharides (tri-, tetra-, and higher oligosaccharides) are represented as a single component, i.e., GOS.

$$E + Lac \underset{k_1}{\overset{k_1}{\rightleftarrows}} ELac \tag{7.4}$$

$$ELac \xrightarrow{k_2} EGal + Glu \tag{7.5}$$

$$EGal \underset{K_3}{\overset{k_3}{\rightleftarrows}} E + Gal \tag{7.6}$$

$$EGal + Lac \underset{k_4}{\overset{k_4}{\rightleftarrows}} E + GOS \tag{7.7}$$

$$E + Glu \underset{k_5}{\overset{k_5}{\rightleftarrows}} EGlu \tag{7.8}$$

where *E, Lac, Glu, Gal, EL, EGal, and EGlu* represent the enzyme, lactose, glucose, galactose, enzyme–lactose complex, enzyme–galactose complex, and enzyme–

glucose complex, respectively, and the k values are the apparent rate constants for the individual reaction step.

7.5.6 Reactor Configuration

Several types of reactors with different modes of operation (batch, continuous, and semi-batch) can be used for the enzymatic synthesis of GOS. The enzymatic reactions may be conducted under soluble (free enzyme) or immobilized states. Soluble state enzyme offers better mixing between enzyme and the substrate leading to better yield of GOS. The major disadvantage is that the enzyme needs to be deactivated after each batch and can't be reused. On the other hand, immobilization of an enzyme offers several advantages such as the enzyme can be used under the continuous mode, enzyme reusability, better enzyme stability, reduction in processing cost, and ease of separation of the enzyme after the reaction. Several supports, such as ion-exchange resin, merckogel, chitosan beads, agarose beads, polymeric beads and membrane, graphite, coconut fiber, cotton cloth, etc. can be used for the immobilization of enzymes. However, the immobilized process offers mass transfer limitation of the reactant toward the enzyme (Palai and Bhattacharya 2013). The modes of operation and reactor configurations may affect the enzyme characteristics and hence the yield of the products (Gosling et al. 2010). The most common type reactors used for the enzymatic GOS synthesis are batch, semi-batch, continuous stirred tank reactor (CSTR) or chemostat, packed bed reactor (PBR), and plug flow reactor (PFR). Among these, the PFR shows higher lactose conversion under immobilized conditions; however, shows a higher pressure drop (Roy and Gupta 2003).

7.6 Recent Advances in GOS Production

The use of purified lactase enzymes for the synthesis of GOS has been studied widely by several authors. The extraction and purification of proteins from microbial sources are a very cumbersome and time-consuming process that requires additional downstream processing steps apart from the culture of the microorganisms. The procedure may not be economically feasible because of the high operating cost. This is probably one of the reasons that hinder the commercialization of the process. In this regard, the utilization of whole cells instead of the purified enzyme could be a useful alternative to minimize the operating cost. Furthermore, GOS formation using whole cells has the additional advantage of the utilization of monosaccharides (glucose and galactose) as a carbon source for cell growth. This will also be expected to enhance the GOS yield. The major concern associated with the use of whole cells that produce intracellular proteins is the diffusional limitation of the substrate through the cell membrane. To facilitate the transport process, various cell

permeabilization agents such as detergent (digitonin and cetyltrimethylammonium bromide) or chemicals (ethanol, propanol, toluene, chloroform, acetone, etc.) may be used (Panesar et al. 2006).

Few studies on the formation of GOS using the whole-cell under free as well as immobilized states have shown comparable yields of GOS (36% to 43% w/w) as compared to purified enzyme system (Goulas et al. 2007; Pham et al. 2019; Roy et al. 2002; Srivastava and Mishra 2019; Yu and O'Sullivan 2018). A pre-incubation step may be introduced during yogurt making process to reduce the overall time for the growth of the cells (Lamoureux et al. 2002). A mixed culture system comprising *Bifidobacterium bifidum* and *Saccharomyces cerevisiae* cells was studied by Goulas et al. (Goulas et al. 2007) for simultaneous formation and purification of GOS. A recent study used mixed yogurt starter cultures containing *Lactobacillus acidophilus*, *Streptococcus thermophilus*, *Bifidobacterium lactis*, *Lactobacillus delbrueckii* subsp. *Bulgaricus,* and *S. thermophiles* for the production of GOS from whey reported a 25.4% GOS yield (Fischer and Kleinschmidt 2021).

Few advancements were also seen in recent times in the immobilization technique. A smart polymer (also known as responsive/intelligent/environmentally sensitive/reversible polymer) can be utilized for the immobilization of enzymes instead of any solid support. The polymers can change their phase reversibly upon application of environmental stimuli (temperature, pH, ionic strength, etc.) and regain their previous phase once the stimuli are removed (Ivanov et al. 2003; Kumar et al. 2007). This will minimize the mass transfer limitations. Thermo-responsive poly-*N*-isopropyl acrylamide (where phase change occurs on changing the temperature around a certain value, known as critical solution temperature) is one such polymer that can be conjugated with lactase enzyme to produce a smart bioconjugate and subsequently be utilized for GOS synthesis. Owing to the unique soluble-insoluble properties of the bioconjugate, the reaction may be conducted under a soluble state. This may overcome mass transfer limitations encountered during the reaction with enzymes immobilized on solid supports (Shakya et al. 2010). Furthermore, the separation of the bioconjugate becomes very easy as it can be done by heating the solution at a temperature above its lower critical solution temperature (Palai et al. 2014a, 2015).

7.7 Conclusions and Prospects

Whey is an abundant waste from the dairy industry. A huge amount of whey is produced annually across the globe as there is a consistent increase in the demand and production of milk products. It contains a considerable amount of proteins, lactose, and minerals. The waste stream may lead to a huge threat to the environment as it has high BOD and COD values. However, whey can be valorized by applying various advanced separation and purification technologies. This will add value to whey (waste) and be considered a potential precursor for several products. Various valuable products such as lactose, WPC, WPI, etc. can be recovered from whey.

Lactose present in whey may be further converted to GOS by the action of lactase. The source of the enzyme has a huge role in the yield and the type of GOS formation.

Most of the studies in this regard are limited to the laboratory scale. The industrial-scale production of GOS from either lactose or whey is not flourished yet due to the unavailability of pilot-scale data. Currently, very few companies across the globe are producing GOS on a commercial scale. Furthermore, the purity of GOS that is being commercialized is another concern. The GOS products currently available commercially have a purity of around 70% which is to be further improved for better nutraceutical properties. There is a need to explore the conversion of whey to GOS directly in a more economical way by proper designing of processes and reactors. Hence, whey valorization is a promising way of its utilization but there is a huge scope for further research.

Competing Interests The authors declare that they have no competing interests.

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Chapter 8
Biorefineries: An Integrated Approach for Sustainable Energy Production

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Abstract Biorefineries represent an integrated approach to facilitate biomass conversion to produce useful fuels, energy, and bulk chemicals from biomass. It employs microbes in the production process, employing methods that have a low carbon footprint. This chapter reviews the various approaches inside a modern biorefinery, from enhanced production of ethanol which can be used as a petrol alternative, to genetic engineering, allowing the production of solvents, bulk chemicals, plastics, and fibers. These advances make production in biorefineries greener as they counter various environmental implications by using lesser energy and being less toxic, producing lesser waste and cost-effective high-end products compared to their traditional manufacturing counterparts. Additionally, the extensive development in applications of biotechnological tools in biorefineries that convert biomass feedstocks to energy and other useful products is also reviewed. From pyrolysis-based processes to gasification, sugar-based biorefineries, and energy crops along with oilseed and lignocellulosic biorefineries. Further, the challenges to integrating higher value chemicals production systems with commodities, for energy and fuel, are also presented, with scope for optimization through resource utilization while minimizing wastes also discussed. Such advances catalyze diversification in feedstocks and products and contribute to sustainability from both economic and environmental perspectives.

Keywords Biomass · Biofuel · Biorefining · Bioprocesses · Sustainability

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Abbreviations

CBH	Cellobiohydrolase
crRNA	CRISPR RNA
DBS	Double-stranded breaks
DME	Dimethyl ether
EG	Endoglucanase
GalP	Galactose permease
GPI	Glycosylphosphatidylinositol
IEA	International Energy Agency
LCF	Lignocellulose feedstock
PEP	Phosphoenolpyruvate
PHA	Polyhydroxyalkanoates
PTS	Phosphotransferase system
RNAi	RNA interference
SSF	Simultaneous saccharification and fermentation
TAG	Triglycerides
TALENs	Transcription activator-like effectors nucleases
ZNFs	Zinc finger

8.1 Introduction

The concept of the biorefinery is based on developing products and energy from bio-based renewable resources. The world has been facing consequential issues like climate change, global warming, pollution, exhausting fossil fuel reserves, and over-population to name a few (Lewandowski 2018; Goswami et al. 2022a). Waste management has become expensive and troublesome (Umar et al. 2021; Chaturvedi et al. 2021). An alternative to the oil economy to fulfill the demand for energy and materials is to imply the bioeconomy, i.e., to produce energy and materials from renewable bio-based resources.

In biorefineries, instead of oil and gas, a plant is used as the carbon source which plays a significant role in the decarbonization of our energy systems and lowering the global emissions, thus keeping in check the impact of carbon dioxide on the global warming (Katakojwala and Mohan 2021; Goswami et al. 2020). Biorefineries also help in reducing pollution by limiting waste and preventing the burning of by-products as they use all the parts of the plant, especially those which are not edible, to produce the desired products. The objective behind developing biorefineries is to grow the economy in a sustainable way with new markets based on products that are renewable and environment friendly. The two broad groups of products of biorefineries are energy (in the form of synthetic biofuels, such as bioethanol and biodiesel) and products (materials, chemicals, feed, and food ingredients) (De Bhowmick et al. 2018; Agrawal et al. 2020; Kumar and Verma 2020a).

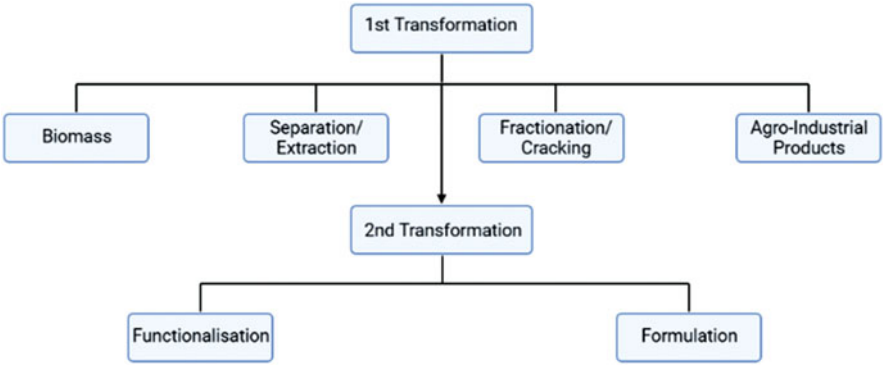

Fig. 8.1 A schematic representation of general process of transformation in a biorefinery

The plants are constituted by a range of different molecules, and each extractable constituent can be utilized according to its potential to develop a certain product. Ninety-five percent of a plant's composition consists of lignin, carbohydrates, fats, and proteins, and the rest of the 5% has constituents like flavors, vitamins, dyes, and other smaller molecules which also possess a high value and can be extracted and utilized in biorefineries. These different components of a plant can give rise to different biorefineries based on the type of carbon source, i.e., sugar (sucrose and starch), carbohydrates, and lignocellulose.

According to the global scheme followed by all the types of biorefineries, there are several steps involved in the transformation of biomass which takes place in two stages as shown in Fig. 8.1. In the first transformation, all the desired components of the plant are first extracted or separated followed by a process called cracking or fractionation in which biochemical or biological technologies are involved. The first transformation leads to the production of the desired molecule. The biomass extracts then undergo another treatment called functionalization which results in the formation of the agro-industrial product. In many industries, these products are considered the intermediates which on further formulation leads to the manufacturing of four different categories of products (Bozell and Petersen 2010; Verma 2022). The most predominant category of the products of biorefineries is energy in the form of biofuels like biodiesel or bioethanol (Rezania et al. 2020). One of the chief applications of the molecules derived from biomass is the production of chemical intermediates called synthons (Shylesh et al. 2017). These biomaterials are said to have a great potential to be used as an alternative to the fossil-derived molecules and may also be used as the bulk in biosynthetic pathways which is why they are the base of modern chemistry. The other categories of bio-based products include polymers and plant fibers. Biopolymers are being used to form the matrix of the composites. Plant fibers can be used as an alternative to the carbon and glass fibers being used in the composites.

The last group of bio-based products deals with the food industries in which the biorefineries are helpful in supplementary production or enhancement of the components.

8.2 Industrial Application of Biotechnology

Biotechnology is one of the greatest technological feats of humans that finds its use in almost every walk of life including agriculture, manufacturing, and healthcare, and is a key tool in achieving sustainable development (Yu et al. 2019). Biotechnology comprises all the techniques that employ living systems or its part, for production purposes. The mystery behind fermented foods and alcoholic beverages that have been in use since ancient times was first unraveled by Louis Pasteur who proved fermentation as a microbial process (Gal 2008). Since then, the fermentation industry has shown tremendous growth and become the primary mode for the production of ethanol and solvents like acetone and butanol. Furthermore, this process has also been utilized for the commercial production of citric acid (the first organic acid, in 1923) and penicillin (the first drug, in 1944).

At present, the socio-economic development of a nation is highly dependent on energy resources. While a majority of approximately 78.4% of energy needs are met using non-renewable fossil fuels; a meager demand is met by renewable sources (Bose et al. 2021a). Earlier due to the availability of petroleum and ease in the chemical process for producing various bulk petroleum-based chemicals like ethanol; the fermentation industry became less prominent. However, with depleting fossil fuel reserves and increasing environmental complications, there has been a gradual shift towards the production of energy and chemicals using fermentation in a biorefinery.

The use of modern biotechnological tools for the sustainable production of energy, chemicals, and materials from renewable sources often known as white biotechnology, ensures low energy consumption and low waste or pollutants released into the environment. Moreover, the microbes are being used for the process of fermentation to convert sugary biomass feedstock to bioalcohol or biohydrogen (Kumar and Verma 2020b). They utilize agricultural lignocellulosic or organic carbohydrate-rich wastes as inexpensive raw materials (Rezania et al. 2020; Agrawal and Verma 2020; Bhardwaj et al. 2021). This makes biotechnological innovations a crucial tool for producing an alternative source of energy.

Apart from the energy industry, biotechnology also finds its application in the chemical and pharmaceutical industries (for drug discovery and production processes). Paclitaxel, a famous anti-cancer drug has been in commercial production using plant cell cultures. Moreover, hybridoma technology has been in use for producing various monoclonal antibodies that can also be used in cancer immunotherapies (Schmid et al. 2018). Fungal and bacterial cells have been utilized in the production of various materials (Agrawal and Verma 2021; Alam et al. 2021). The

filamentous fungal mycelium can be converted to bio-based solid composites, foam, or leather-like non-woven fabrics.

Food security is also a major concern among nations. Having just enough food to meet the demand of a rapidly increasing population, especially for a country like India, is a daunting task along with limited arable land resources, food versus fuel, fodder, or other crop resources dilemma, and increased pressure to reduce negative environmental effects of conventional agriculture (Bose et al. 2021b). Hence, it is inevitable that the modern-day agriculture and food industry are too influenced by advances in biotechnology. For example, genetic engineering has made possible the development of transgenic crops and animals that has ensured high farm productivity. Cultured meat or plant cells provide a lucrative option as a healthy, protein-rich food raw material.

Biotechnology has influenced a lot of industries in a good way, and it will continue to be a medium for sustainable industrial production (Bose et al. 2020a). These advances in biotechnologies have ensured a greener production in biorefineries as they counter various environmental implications. They have done so by using lesser energy and being non or less toxic, producing lesser waste compared to their traditional manufacturing counterparts. Biorefineries have taken an integrated approach to ensure the conversion of complete biomass into energy, fuel, chemicals, or high-end products in an environmentally sustainable and cost-effective way.

8.3 Biorefinery Principles

Biorefinery is a process of converting biomass to energy, transportation biofuel, and other marketable bioproducts like drugs, biochemicals, and biomaterials by combining various technologies (Bozell and Petersen 2010). These technologies work by breaking the biomass resources (wood, husk, food waste) into their building blocks (protein, carbohydrate, TGA, etc.) for conversion into value-added products (Lewandowski 2018). The microbes and the biomass feedstock, which have been used for the production of a bioproduct, essentially form the main principles behind a biorefinery.

8.3.1 Role of Microbes

Inside a biorefinery, the biomass can be converted to a bioproduct by one of the three pathways, namely thermochemical, biochemical, or physicochemical pathways. However, a combination of these pathways can be integrated into a multi-staged biorefinery. The thermochemical route involves high temperature, pressure, and reaction rates for the conversion processes and so is quite an energy expensive. It includes methods like pyrolysis and gasification. Gasification involves treating

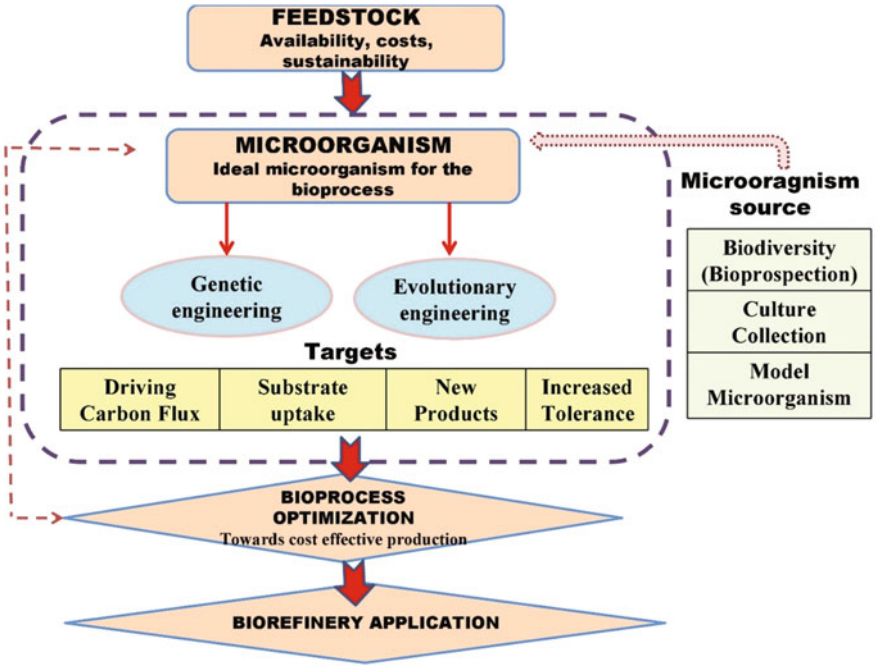

Fig. 8.2 Proposed stepwise development of industrial bioprocesses for biorefineries, which can act as mediators to improve the efficiency of microbial processes

biomass at high temperatures (>700 °C) and low oxygen concentrations to produce syngas (which is basically a mixture of H_2, CO, CO_2, and CH_4) that can be directly used as biofuel (Lewandowski 2018). On the other hand, pyrolysis involves intermediate temperatures (300–600 °C) in lack of oxygen for conversion of biomass feedstock into a liquid pyrolytic bio-oil.

Whereas the physicochemical route involves a mix of physical and chemical processes requiring lesser energy than the thermochemical one. The production of biodiesel from oilseeds is an example of the physicochemical process as it involves oil extraction by manual pressing or solvent extraction followed by transesterification of oil. Both these processes have various environmental concerns like increasing pollution by emission of toxic gases and wastes or global warming. The falling quality of the environment necessitates the use of alternative modes of industrial production. This can be achieved using the bioconversion option or the biochemical processes. Since the discovery of microbes being responsible for fermentation, humans have been able to use this process to their advantage for various industrial applications. Biorefineries employ the metabolic and enzymatic properties of microorganisms for the conversion of biomass feedstock into a bioproduct by biochemical processes like fermentation, aerobic and anaerobic digestion (Bose et al. 2020b). Figure 8.2 highlights the strategies that can be implemented to achieve optimized bioprocesses within biorefineries.

The choice of a suitable microbe (bacteria, fungi, or algae) for a fermentation process depends on the product required, the substrate used, and the growth requirements of the chosen microbes (Arora et al. 2021). Microorganisms like *Escherichia coli*, *Corynebacterium glutamicum,* and *Saccharomyces cerevisiae* are among the important producers used in industries (Regassa et al. 2021). The use of natural strains for ensuring maximum production yield is quite challenging as the yield will be highly influenced by variations in pH, temperature, and pressure, their inability to use all substrate components, product and substrate toxicity, and contamination. Therefore, selecting a microbe having all the necessary traits is necessary and has been made possible by successful genetic and metabolic engineering techniques (Yu et al. 2019). These techniques have made possible the development of microbial strains having high productivity by improving stress tolerance to inhibitory compounds, increasing substrate uptake range, and driving carbon flux towards the desired pathway.

Several strains of yeast have been developed and are in use for the production of bioethanol and other bioalcohols or in brewer and baker's industries. Also, *E. coli* has been modified to produce several biochemicals like threonine, ethanol, butanol, and succinic acid (Zhang et al. 2018). The microbes have been developed such that they can use a variety of feedstocks as substrates including carbohydrate-rich biomass, oil-based and lignocellulosic feedstocks. Furthermore, as more and more new microbes, pathways, enzymes, and genes are getting discovered, they are being employed for new productional processes like *Pichia ciferrii* has been recognized for its potential of producing sphingolipids including sphingosine (Börgel et al. 2012). Microorganisms thereby play an important role in the development process of a biorefinery.

8.3.2 Biomass Feedstock

The overexploitation of fossil fuels as a prime source of energy and chemicals raises several societal and environmental concerns. Also, they are getting exhausted at a far greater rate than they are replenished by the natural carbon cycle. Therefore, there is a need to look at alternative renewable sources. The organic matter often called biomass is a promising feedstock that can be used in biorefineries (Bose et al. 2021c). Compared to the traditional fossil fuels like petroleum resources that are localized only in certain countries, biomass is abundant and cheaply available in most non-industrialized nations. Moreover, since they are synthesized because of biological processes (like photosynthesis in plants that converts atmospheric carbon and water to sugars which are later used for the synthesis of complex material called biomass), a consistent, renewable, and regular supply of feedstock can be ensured (De Bhowmick et al. 2018). While there are several classifications for biomass feedstock, they are mainly composed of the following biomass components: triglycerides, proteins, nucleic acid, carbohydrates, and lignin (Rezania et al. 2020). They can be obtained from 4 different sectors, namely

1. Agriculture for dedicated energy crops and residues,
2. Forestry for wood or other lignocellulosic wastes,
3. Industries (for processed residues and leftovers) and households (for municipal solid wastes and wastewater),
4. Aquaculture (includes algae and seaweeds).

8.3.2.1 Sugar or Starch-Rich Crops

Carbohydrates like starch, sucrose, or other monosaccharides like glucose, mannose, and galactose present in the crops are the main raw materials for production in the biorefinery. Almost 90% of today's ethanol production is based on the fermentation of biomass feedstock obtained from sugar crops (like sugar cane and beet) and starch crops like corn (Detroy 2018), which is integral to biorefinery operations.

To obtain a higher yield of products in biorefinery from these agricultural feedstocks, certain pretreatment steps are necessary. Starch being a polymer of hundreds or thousands of glucose units needs to be broken down into mono or disaccharide units to be employed for fermentation. This can be achieved by the use of starch digesting enzymes like α-amylase, β-amylase, and glucoamylase (Xu et al. 2016).

8.3.2.2 Lignocellulosic Biomass

Being a major source of food and animal feed, the starch and sugar-rich crops present a dilemma of food vs energy. Thereby, raising serious concerns about food security and hindering the prospects of their utilization in biorefineries as feedstocks. This can be overcome by the use of lignocellulosic biomass which is the most abundant biomass component found in all plant-derived materials and residues like straw and wood wastes from agricultural, forestry, or industrial sectors and municipal solid wastes (Umar et al. 2021). Lignin (15–25%), cellulose (30–50%), and hemicellulose (20–40% of total feedstock dry matter) are the three major components of lignocellulosic biomass (Katakojwala and Mohan 2021; Bhardwaj and Verma 2021). While cellulose and hemicellulose are essentially polysaccharides, lignin is the largest non-carbohydrate portion of lignocellulose that is made of phenolic polymers and acts as a glue providing rigidity to the plant's and tree's structures.

The structure and properties of lignin suggest its use as a feedstock for the production of supramolecular materials and aromatic compounds. Moreover, the carbohydrate fraction of this biomass, i.e., cellulose is composed mainly of D-Glucose units liked by β-glycosidic bonds (C-6) and hemicellulose is a complex heteropolysaccharide with xylose and mannose as main units (Xu and Ferdosian 2017). These sugar derivatives can be employed to produce a wide range of organic acids like glutamic, succinic, fumaric, and aspartic acids that are useful starting materials in various chemical industries (Werpy and Petersen 2004).

8.3.2.3 Industrial and Municipal Wastes as Biomass Feedstock

Wastes from industrial and municipal effluents can pose a serious pollution problem. However, they are also an abundant source of biomass that can be used as feedstock in biorefineries. This will have a dual effect, i.e., benefit to environment and manufacture of chemicals and other products in biorefineries (Shylesh et al. 2017; Goswami et al. 2021). The wet milling or corn refinery industry generates a significant number of useless by-products like corn fibers. Moreover, the dairy industry produces cheese whey as waste that contains about 7% total solids (comprising 70% lactose and 13% proteins). Both act as abundant inexpensive resources that can be utilized in fermentation biorefineries to produce chemicals and fuels like polyhydroxyalkanoates (PHAs) from whey (Amaro et al. 2019). Furthermore, municipal solid wastes and animal manure can be employed in the production of biogas (Bose et al. 2020a).

8.3.2.4 Lipids, Proteins, and Nucleic Acids

Vegetable oil from oleaginous plants like soybean, palm, or rapeseed and waste cooking oils can be used as a feedstock for the production of biofuels. Lipids produced by these plants and other oleaginous microbes mostly exist as triglycerides (TAG) and free fatty acids that are converted to biodiesel by a process known as transesterification (Rathore et al. 2016). Along with biofuel, these oil and fats can be employed as feedstock for the production of various oleochemicals, lubricants, coatings, glycerol, surfactants, cosmetics, and other products.

On the other hand, biomass also contains a small fraction of proteins and nucleic acids that are used as nitrogenous sources for the growth of the cell, but these components do not act as a potential feedstock for the production of fuel and chemicals in biorefinery.

8.4 Classification of Biorefineries

Following are the major and most popular types of biorefinery:

1. Conventional biorefinery: It stands on existing industries like the starch and sugar industry.
2. Green biorefinery: It uses herbaceous wet biomass like immature cereal, green grass, clover, or alfalfa.
3. Lignocellulosic feedstock biorefinery: It uses nature-dry biomass containing cellulose, lignin, or hemicellulose.
4. Two-platform concept biorefinery: It comprises two types of platforms, i.e., the syngas platform and the sugar platform.

5. Whole-crop biorefinery: It uses an entire crop as raw material, such as cereal grains.
6. Marine biorefinery: It stands on marine biomass such as seaweeds or microalgae.
7. Forest-based biorefinery: It fully integrates biomass and other feedstocks for concurrent production of chemicals, fibers, pulp, and energy.
8. Thermochemical biorefinery: It utilizes entire biomass using several thermochemical methods.
9. Liquid phase catalytic processing biorefinery: Based on the conversion of intermediates derived from biomass in the liquid phase into value-added products.

The share of biofuels in the transportation industry has been on the rise, serving as the key force behind the breakthrough development of modern and advanced technologies or processes to produce gaseous and liquid biofuels in biorefineries. In the year 2020, according to the directive of renewable energy in Europe, the target set for biofuel was 10% and by 2030, Inter-governmental Panel on Climate Change and IEA expect a 10–20% contribution of biofuels to the transportation industry worldwide (Kolosok et al. 2021). Several acquisition programs have been established for the products derived from biomass, but no specific targets have been set for them. That being so, the development and advancement of energy-driven biorefineries stand on economical and efficient production of biofuels for transportation, with concurrent production of biomass-derived materials and chemicals which will be beneficial for the economy as well as the environment.

Further, the approach to the classification of biorefineries is based on using the following four attributes for the classification of an individual biorefinery system:

1. Platforms
2. Feedstocks
3. Processes
4. Products

In a biorefinery system, the feedstock is converted into products through a pathway involving processes and platforms where the platforms serve as the intermediates resulting in the final products and hence are considered the key feature while classifying biorefineries (Diep et al. 2012).

8.4.1 Platforms Recognized in Energy-Driven Biorefineries

Platforms are described as the intermediates that act as a bridge between the raw material and the final products. This concept is the same as in the petrochemical industry where the crude oil is processed to produce several intermediates which are then functionalized into the final products. The raw materials undergo several different conversions to form the intermediates and for this reason, the platforms are said to be the main “pillars” for the classification of biorefineries (Wenger and

Table 8.1 Features of biorefineries and their classification for sustainable bioenergy production

Platforms	Feedstocks	Processes	Products
1. Syngas 2. Biogas 3. Electricity and heat 4. Oils 5. Hydrogen 6. Organic juice 7. Lignin 8. Pyrolysis water 9. C5 sugars C6 sugars	1. **Crops** (a) Grasses (b) Starch crops (c) Sugar crops (d) Oil crops (e) Lignocellulosic crops (f) Marine biomass 2. **Residues** (a) Oil-based restudies (b) Lignocellulosic residues (c) Organic residues and others	1. **Physical/Mechanical** (a) Extraction and separation (b) Pretreatment (c) Pressing/disruption (d) Mechanical fractionation 2. **Chemical Processes** (a) Hydrolysis (b) Catalytic processes (c) Water electrolysis (d) Hydrogenation (e) Esterification (f) Steam reforming (g) Water-gas shift reaction 3. **Biochemical processes** (a) Fermentation (b) Enzymatic conversion (c) Aerobic/anaerobic digestion 4. **Thermochemical** (a) Pyrolysis (b) Combustion (c) Hydrothermal upgrading (d) Gasification	1. **Material** (a) Biomaterials (b) Polymers & Resins (c) Food & feed (d) Chemicals (e) Building blocks (f) Fertilizers 2. **Energy** (a) Bioethanol (b) Synthetic biofuels (c) Biodiesel (d) Biomethane

Stern 2019). Table 8.1 shows the approach for classifying product streams for biorefinery operations.

Major platforms recognized in the energy-driven biorefineries are as follows:

- Syngas (a mixture of carbon monoxide and hydrogen) is obtained from coal gasification.
- Biogas (consisting of carbon dioxide and methane) is produced by the anaerobic digestion of organic matter.
- Electricity and heat are used as the energy source in biorefineries or sold to the grid.
- Oils (triglycerides) from algae, oilseed crops, and oil-based residues
- Hydrogen H_2 is obtained from fermentation, steam reforming, water hydrolysis, and water-gas shift reaction.
- Organic juice is obtained by pressing wet biomass, e.g., grass.

- Lignin (phenylpropane building blocks) is obtained through the conversion of lignocellulosic biomass.
- Pyrolysis water (consisting of several components of different sizes)
- C5 sugars ($C_5H_{10}O_5$; arabinose, xylose, etc.) are obtained from food and feed side streams and through hydrolysis of hemicellulose.
- C6 sugars ($C_6H_{12}O_6$; galactose, fructose, glucose, etc.) are obtained through the hydrolysis of starch, sucrose, hemicellulose, and cellulose.

8.4.2 The Feedstock Used in Biorefineries

A feedstock is the unprocessed or raw, renewable material, also called biomass, that is used to produce energy, intermediate products, or finished products in a biorefinery. It is an integral part of the biorefinery system. Different types of feedstocks can produce a wide range of products by undergoing different conversion processes in a biorefinery. The biomass feedstock can be classified as primary, secondary, and tertiary feedstock (Diep et al. 2012). Primary feedstock is the unprocessed raw material that undergoes several conversion processes to produce the finished or intermediate product. A secondary feedstock is obtained as residue from the processing of primary feedstock, and tertiary feedstock is the residue obtained at the end of the secondary treatment (Werpy and Petersen 2004).

Today, four major sectors contribute the renewable organic biomass for biorefineries, namely

1. Forestry (logging residues, wood, short-rotation poplar)
2. Agriculture (dedicated crops and crop residues)
3. Aquaculture (Algae, seaweed, etc.)
4. Industrial and domestic activities.

The composition of biomass feedstock varies with different proportions of basic components like lignin, cellulose, starch, hemicellulose, proteins, and triglycerides. Three major chemical elements found in the composition of biomass feedstocks are carbon, hydrogen, and oxygen with smaller quantities of sulfur, nitrogen, and ashes (Xu and Ferdosian 2017). Besides these components, biomass feedstocks also consist of some water content. Specific volume and heating value are the two main characteristics that are considered for the composition of biomass feedstocks (Wenger and Stern 2019). Based on their composition, the biomass feedstock can be classified as follows:

Dedicated Feedstocks

1. Starch crops, e.g., corn, wheat, maize, sweet sorghum
2. Sugar crops, e.g., sugarcane and sugar beet
3. Oil-based crops, e.g., palm oil, rapeseed, soya, Jatropha curcas
4. Lignocellulosic crops, e.g., wood, switchgrass, short-rotation poplar, Miscanthus

5. Marine biomass, e.g., seaweed, microalgae, and macroalgae
6. Grasses, e.g., grass silage, green plant materials, plant shoots, and immature cereals

Residues

1. Lignocellulosic residues, e.g., sawmill residues, crop residues like straw and stover, etc.
2. Oil-based residues, e.g., used cooking oil from restaurants and households, animal fat from food industries, etc.
3. Organic residues, e.g., manure, organic urban waste, wild fruits, crops, etc.

8.4.3 Conversion Processes Used in Biorefineries

Biorefineries account for a wide range of conversion processes that concentrate different types of biomasses into one or many different products. The biomass feedstock must undergo different conversion processes to produce marketable products like biochemicals (such as enzymes), fuel, biomaterials, food, or feed (Corona et al. 2018). Depending on the type of products, biorefineries can be classified as systems in which the processes like separation or fractionation into polymeric products like biomaterials, feed, or food are the main operations, and systems for biochemicals and biofuels in which processes like depolymerization and/or chemical, biochemical, or thermochemical processes are employed.

In a biorefinery, to produce biofuel, the components of biomass are depolymerized followed by deoxygenation. Deoxygenation is considered a crucial step in the production of transportation biofuels as the presence of oxygen molecules may result in a reduction of the heat content of the molecules giving them a higher polarity, thereby making the blending of biofuels with existing fossil fuels a little difficult (Gunukula et al. 2018). On the other hand, in chemical products like polyols and organic acids, the presence of oxygen is considered useful as it adds more value to the chemical and physical properties of the compound.

According to the International Energy Agency (IEA), based on the type of conversion processes, a biorefinery can be classified into four major groups:

1. **Physical or mechanical,** e.g., distillation, pressing, milling, pretreatment, and separation. In this group of biorefineries, the feedstock components are separated, and/ or their size is reduced, and the biomass does not undergo many changes in its chemical structure.
2. **Chemical,** e.g., pulping, hydrogenation, transesterification, hydrolysis, and oxidation. These processes lead to changes in the substrate.
3. **Biochemical,** e.g., fermentation, anaerobic digestion, enzymatic conversion, etc. These processes take place with the help of enzymes or microorganisms under mild conditions, i.e., low temperature and pressure.

4. **Thermochemical,** e.g., gasification, combustion, pyrolysis, and hydrothermal upgrading. These processes may take place with or without catalytic means, under extreme conditions like high temperature and pressure.

A common depiction between different types of biorefineries is the one between biochemical and thermochemical pathways (Cherubini et al. 2009). The major processes within these groups of biorefineries are fermentation and gasification. The major components of feedstocks used in biorefineries are starch, sugar, and lignocellulose (woody biomass). Sugar and starch are comparatively easier to ferment and only the cellulosic and hemicellulosic parts of wood can undergo changes and made available for fermentation, whereas the rest of the parts, including lignin, are to be gasified. Ethanol is the major product of fermentation while several other substances, such as methanol, dimethyl ether (DME), hydrogen, and methane are produced because of the gasification pathway (Yu et al. 2019). Additional materials and energy are required for all these processes to take place. The futuristic approach is to reduce the use of auxiliary materials and energy and utilize inputs from renewable sources like residues from processed biomass, solar photovoltaics, and hydropower.

8.4.4 Products of Biorefineries

Biorefinery concerns with the utilization of biomass to produce high-value chemicals, heat, electricity, fuels, and many other important bio-based products through different conversion processes (Zhang et al. 2018). Green chemistry companies have already been producing several traditional bio-based products in the biorefineries such as liquid fuels biodiesel and bioethanol, certain organic acids like acrylic acid and lactic acid, and natural sweeteners such as xylitol and sorbitol (Detroy 2018). Besides these, biorefineries can produce several other by-products using renewable biomass feedstock such as biopolymers, nutritional yeasts, biopigments, and biosurfactants (Cherubini et al. 2009).

Biorefineries can produce both energy-based and non-energy-based products and are mainly categorized under two broad groups:

- **Energy-Driven Biorefinery System**: In these biorefineries, the biomass feedstock is generally utilized to produce secondary energy carriers such as heat, electricity, and transportation biofuels (Lewandowski 2018). The residues from conversion processes are either sold or upgraded to bio-based products of high value which provide benefits to the economy and the environment.
- **Material-Driven biorefinery System:** These biorefineries mainly deal with the production of bio-based products such as high-value chemicals, biomaterials, food, feed, and lubricants (Cherubini et al. 2009). These biorefineries approach generate power or heat from the residues of the conversion processes which could be sold or kept for internal use.

However, a few products like bioethanol or biohydrogen can be used as both energy carriers and as the material products. Besides heat and power, energy-driven biorefineries also produce up-and-coming transportation biofuels such as biodiesel, bioethanol, biomethane, and synthetic biofuels; through contemporary processes that are much faster than the geological processes of formation of fossil fuels (Mona et al. 2020). Bioethanol is an alcohol produced by fermentation from starch or sugar crops like sweet sorghum, corn, or sugarcane. Cellulosic biomass from non-edible sources, such as grasses and trees, is also being used as feedstock to produce ethanol (Rezania et al. 2020). Biodiesel is produced through the process of transesterification from fats and oils and can be used as a transportation fuel for vehicles in its pure form, but it is usually used as an additive to diesel in the diesel-powered vehicles to reduce the levels of particulate matter, hydrocarbons, and carbon monoxide (Singh et al. 2020). The bio-based material products mainly include high-value chemicals which find their applications in pharmaceutical and/or food industries, animal feed, and fiber products. following are some major subgroups of bio-based material products produced in a biorefinery:

- Biomaterials, e.g., pulp and paper, fiber products, panels, polysaccharides, etc.
- Biohydrogen
- Polymers and resins, e.g., PLA, PHA, resins.
- Chemicals and building blocks, e.g., amino acids, phenols, furfural, polyols, xylitol, fine chemicals, aromatic compounds, lactic acid, succinic acid, itaconic acid, and levulinic acid
- Animal feed
- Food products
- Fertilizers
- Glycerin

8.5 Advances in Biotechnological Tools Within Biorefineries

Conceptually, both traditional and novel biochemicals and biofuels can be synthesized in a biorefinery. However, poor industrial implementation has been observed owing to cost competitiveness and low product yield in such productions. Modern techniques like metabolic engineering, genetic engineering, cell surface engineering, and enzymatic pretreatment of feedstock are a few recent advancements in biotechnology that can prove to be a wonderful asset in the realization of the biorefinery concept (Börgel et al. 2012). The influence of products of a biorefinery on the market and thereby on the economy is shown in Fig. 8.3. Such influences are key to the growth of bioenergy in the shadow of fossil fuel-powered economies.

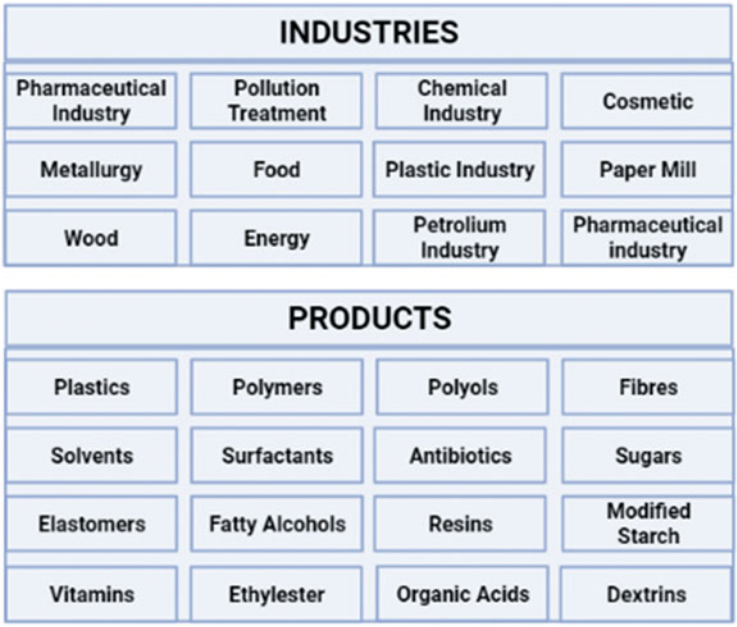

Fig. 8.3 Products of biorefineries and their applications in the economic sectors

8.5.1 Metabolic Engineering

Microorganisms like *E. coli* and *S. cerevisiae* provide an easy platform for the manipulation of metabolism by optimization of the genetic and regulatory processes in the cells for increased production of certain novel substances (Zhang et al. 2018). The process is known as metabolic engineering, and it is a key pillar in the progress of biorefineries. It may involve blocking the competitive pathways or overexpressing relevant genes in microbes for the biosynthesis of required products to improve their yield. This can be used for expanding the feedstock spectrum for production in a biorefinery to include renewables like polymers, complex substrate mixtures, and diluted waste streams that are normally not metabolized by naturally occurring microbes (Börgel et al. 2012). This typically holds true for traditional sugar-based bioprocess microbes like *E. coli*, *C. glutamicum,* and *S. cerevisiae*. Therefore, there is a need to engineer these microbes for harvesting their full production potential in a biorefinery chain to adapt them to various feedstocks other than the traditional glucose substrate.

Naturally, *E. coli* can grow in a variety of monomeric carbon sources like sugars, sugar alcohols, and organics acids except for sucrose (only a few strains have ability to grow in it). Moreover, they lack enzymes for the metabolism of polymeric carbon and their growth is correlated with unfavorable overflow metabolism and catabolite repression. These drawbacks need to be addressed by metabolic engineering of *E. coli* to enable assimilation of complex carbon substrates and fermentation of

sugar polymers. Sucrose metabolizing strains of *E. coli* have been engineered for succinate production via heterologous expression of genes that encode sucrose transporter (srcA), sucrose-6-phosphate hydrolase (scrB), and corresponding regulator (scrR). The alternative strategy of engineering sucrose metabolizing microbe includes the expression of heterologous β-fructofuranosidase (Mohamed et al. 2019). Also, overflow metabolism of acetate secretion as a by-product driven by the intense formation of pyruvate from phosphoenolpyruvate (PEP) via phosphotransferase system (PTS) can be controlled by deleting the PEP dependent uptake system followed by simultaneous overexpression of galactose permease (GalP) that acts as an alternative transporter, reducing acetate accumulation and improving production yield without impairing growth. At last, carbon catabolite repression poses a challenge when using complex substrate mixtures as a feedstock since it is required that instead of sequential carbon source utilization, the *E. coli* strain used, metabolizes the complex carbon substrates in the mixture at the same time. Simultaneous co-fermentation of glucose, arabinose, and xylose can be made possible by deleting ptsG gene that encodes glucose-specific E11 A^{glc} compound of PTS involved in catabolite repression (Buschke et al. 2013; McCloskey et al. 2018).

Metabolic engineering has also made it possible for engineering microbial strains that can utilize lignocellulosic biomass which is otherwise not naturally metabolized. For example, *S. cerevisiae* strain can be engineered for assimilation of xylose which happens to be the second most abundant sugar in biomass via heterologous expression of xylose isomerase, xylose reductase, and xylitol dehydrogenase or overexpressing endogenous GRE3 (Buschke et al. 2013). Additionally, recent advances have made it possible, to mimic the biological pathways in a microbial cell factory leading to increased biosynthesis of novel compounds. This way even plant secondary metabolites like morphine and codeine can be synthesized by the introduction of product-specific metabolic pathways in the engineered microbes. For instance, fermentative opiate production in *E. coli* has been investigated and was found to produce 300 folds higher amount of thebaine (an important raw material in opioid production) compared to reported yeast systems. This can be attributed to a higher activity of enzymes SalS (Salutaridine synthase), SalR (Salutaridine reductase), and SalAT (Salutaridinol 7-O-acetyltransferase) involved in thebaine synthesis from (R)-reticuline (Nakagawa et al. 2016).

Improvements in analytical techniques involved in metabolic engineering like omics and metabolic flux analysis have made it possible to obtain information about gene expression levels and metabolic fluxes. The analyzed results of the multi-omics data can be used to generate mathematical models which can be helpful in studying genetic perturbations and metabolic shifts.

8.5.2 Genetic Engineering

Novel nucleic acid-based genome editing tools such as RNAi (RNA interference) system, ZNFs (Zinc finger nucleases), TALENs (Transcription activator-like

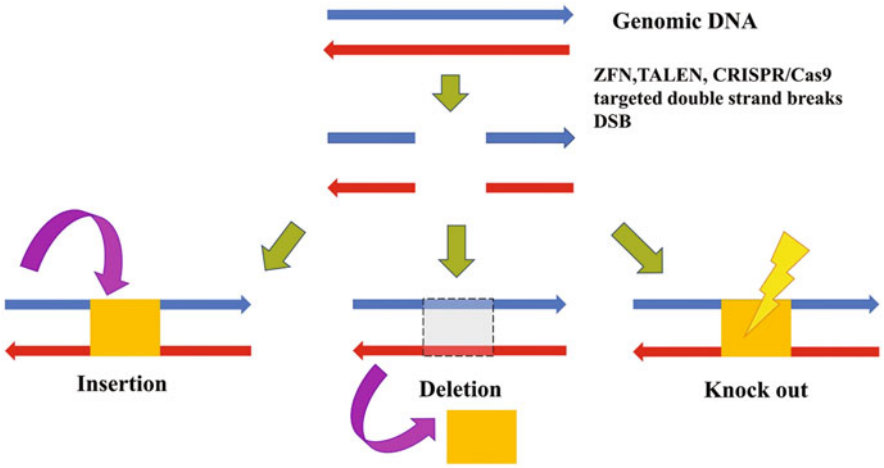

Fig. 8.4 General representation of genome editing via tools like TALEN, CRISPR/Cas9, and ZFN. Target genome can be modified and optimized by inducing mutations like adding desired genes, deleting it, or knockdown of target gene (Adapted from Fayyaz et al. 2020)

effectors nucleases), and CRISPR/Cas9 can be employed for enhancing the productivity and performance of microbial strains, coping with genetic inadequacies, and making them economically competitive for use in biorefinery (Fayyaz et al. 2020). As shown in Fig. 8.4, genetic engineering can include gene knockdown, gene knockout, other modifications like insertion, deletion, or other mutations, and allowing site-specific nucleases to facilitate reverse genetics and integration of transgene by introducing double-stranded breaks.

A reverse genetic approach, RNAi, is used to analyze genes and their functions by targeting the corresponding mRNA molecule. The RNAi targets complementary mRNA sequences rendering them unavailable for translation thereby silencing a particular gene sequence. The technique has been used to identify and characterize several microalgal genes and to study the effect of nitrogen stress on lipid biosynthesis. Lipid accumulation has been increased in diatom *Phaeodactylum tricornutum* by knocking down the gene encoding nitrate reductase via using RNAi system (Levitan et al. 2015).

Another way of improving microbial strain performance in biorefinery is by using nucleases: ZFNs and TALENs. ZFNs are programmable sequence-specific protein-based nucleases derived from FokI endonuclease of *Flavobacterium okeanokoites* which cleaves the target DNA inducing double-stranded breaks (DBS) that are repaired by a DNA repair mechanism. The ZFNs have been introduced in mutated *Chlamydomonas* cells having a cop3 gene into an aphVIII marker which rendered the marker non-functional. The introduction of ZFNs cleaved the gene, thereby restoring the function of aphVIII marker. Like ZFNs, TALENs are another set of effector protein nucleases that were discovered during studies of phytopathogenic bacteria from the genus *Xanthomonas*. Their DNA binding capability mimics the

transcription factors of eukaryotes, hence, can be used for target gene activation (Fayyaz et al. 2020).

CRISPR/Cas9 is the most advanced, efficient, fast, and precise genome editing tool available that can be used for programming microbes specifically for a biorefinery. Originally, CRISPR/Cas system is bacteria's immune mechanism against viral incursions. The Cas9 protein of this system exhibits endonuclease activity which is guided by crRNA (CRISPR RNA) to the target sequence producing double-stranded breaks. This forms the basis of genome editing using this technique and has been utilized to add the omega-3 fatty acid desaturase (fad3) gene which showed a high accumulation of lipid content (46% w/w/) in microalgae *Chlorella vulgaris* FSP-E (Fayyaz et al. 2020).

8.5.3 Cell Surface Engineering

A new technique by which proteins can be expressed on the cell surface using GPI (glycosylphosphatidylinositol) anchor proteins has been developed that provides means for designing simultaneous saccharification and fermentation processes (SSF) for using complex feedstocks like lignocellulose. The technique has made possible the development of yeast strains armed with the expression of various cellulolytic enzymes on the cell surface. For instance, researchers have successfully designed a novel *S. cerevisiae* strain that expresses three types of fungal cellulases: endoglucanase (EG), cellobiohydrolase (CBH), and β-glucosidase (Matano et al. 2012). The strain could improve the conversion of lignocellulosic rice straw biomass to ethanol by 1.4 times when used in combination with commercially available cellulase enzyme (Amoah et al. 2019). By producing microbial strains expressing enzymes on their cell surface, it is possible to develop strains that can metabolize diverse complex feedstocks.

8.6 Biorefineries and Petrochemical Refineries

Sustainable economic growth implies the use of safe, long-term industrial resources (Katakojwala and Mohan 2021). Completely new approaches in research and development, production, and economics are required for the future re-arrangement of a large economy to biological raw resources. Biorefineries combine these technologies required for processing biological raw materials, industrial intermediates, and finished goods. The following are the main targets in the development of biorefineries: product mix (biomass) feedstock mix + process mix (Yu et al. 2019). The combination of biotechnological and chemical conversion of substances will be crucial in this case.

A biorefinery is a facility that processes biomass into a variety of products at the upstream, midstream, and downstream levels. A biorefinery can utilize wood, crops,

organic wastes (both plant and animal-derived), forest residues, and aquatic biomass from forestry, agriculture, and aquaculture, as well as residues from industry and households (algae and seaweeds). Biorefineries are not a new concept. Many traditional biomass conversion processes, such as sugar, starch, and the pulp and paper industry, can be classified as biorefineries to some extent. Several economic and environmental factors, such as global warming, energy conservation, supply security, and agriculture legislation, have pushed those businesses to improve their operations in a biorefinery manner. Three biorefinery systems are currently being researched and developed (Lewandowski 2018). The first is the whole-crop biorefinery, which employs grains or maize as raw materials. Second, there's the green biorefinery, which makes use of naturally moist materials. biomass (e.g., green grass, lucerne, clover) or immature biomass (e.g., cereal). The third type of biorefinery is the lignocellulose feedstock (LCF) biorefinery, which employs naturally dry raw materials like cellulose biomass and wastes.

By adding value to low-value by-products that have limited application in the fuels business, the petrochemicals industry has grown out of oil and gas processing. Plastics, synthetic rubber, solvents, fertilizers, medicines, additives, explosives, and adhesives are among the many important goods produced by the sector. These materials play a vital role in almost every aspect of modern society. Cars, packaging, home goods, medical equipment, paints, textiles, and building materials are just a few of the major uses for petrochemical products (Diep et al. 2012). The petrochemicals industry gets raw materials from refining and gas processing and uses a variety of chemical process technologies to transform these raw materials into marketable products. As raw materials, a variety of feedstocks are used, with cost being the primary motivator. If the inexpensive feedstock is accessible, there will always be someone willing to try to earn a profit by turning it into something useful. These feedstocks are put through a series of operations to produce a small number of chemical building blocks. To generate the final petrochemical products, these building blocks are further processed through several processes.

The following are the advantages of a biorefinery over an oil refinery:

- Biorefineries have a higher capacity and configuration flexibility than conventional refineries.
- A biorefinery selects products based on a variety of parameters, including the composition of raw materials, processing capacity, equipment, and market demand.
- Operating and product diversity allows the biorefinery to adjust production over time and insulate it from economic downturns and seasonal demand cycles.
- If properly constructed, a biorefinery can have a positive impact on climate change, energy security, and rural development.

Several reports and papers have explored the possibility of producing chemicals and polymers from biomass. The US Department of Energy published a report in 2004 that named 12 chemicals that is believed to be future building blocks (Werpy and Petersen 2004). In 2010, this list was reviewed and updated. Transportation biofuels are generally difficult to produce economically. Chemical, material, food,

and feed coproduction can provide the essential added value. A report was recently published the all highlighted bio-based chemicals with immediate potential as "value-added products" in biorefineries. Traditional petrochemical refineries and biorefineries are constantly compared. However, integrating biomass processing in traditional refineries as a strategy to enhance traditional refineries may still be highly appealing, and thus represents a modern systems version of a retrofit problem. The production of "green biodiesel," the NExBTL process, and the catalytic cracking of pyrolytic lignin are only a few examples. Green biodiesel (Petrobras/H-BIO, UOP with ENI) is routinely created by hydrogenating plant oils (or animal fats) with hydrogen from the refinery (Tran et al. 2016).

8.7 Goals and Scope of Biorefineries

Biorefining is a promising method for co-producing bioenergy and bioproducts to improve the benefits and sustainability of petroleum-based products (Amoah et al. 2019). Although various feasibility studies have been undertaken with certain valuations or materials, a full examination of the integration of bio-based products with sustainable bioenergy production is required. System boundaries, technical level, allocation, environmental issues, and unpredictability are determined to be particularly critical challenges. Biorefining is a critical component of the global economy since the diverse range of biomass resources provides many possibilities for a diverse product portfolio to meet society's various demands. Some biorefineries, including those in the pulp and paper business, biofuel sector, and food industry, are currently operating on a commercial scale (De Bhowmick et al. 2018). In addition, new innovative biorefineries are being developed continually.

- The production of energy and materials in tandem (e.g., gaseous or liquid biofuels, chemicals, food, and feed).
- A method that combines numerous steps (e.g., mechanical processes such as pressing and thermochemical processes such as gasification).
- The utilization of a variety of raw materials, both virgin and residual.

The goal of the biorefinery is to maximize or optimize the economic, environmental, and social benefits by utilizing all synergies for sustainable and efficient production. The predicted future growth of the biofuel market, as well as the development of novel biofuel production techniques, needs the construction of new integrated biorefineries. Biomass conversion plants will require plant principles like today's chemical plants or crude oil refining. Integration of innovative biorefinery concepts into existing industrial complexes has promising implications, such as lowering the capital costs of biofuel production plants and thereby lowering chemical analog costs (Ghodke et al. 2021).

As shown in Fig. 8.5, biorefineries must integrate feedstock as an intrinsic element of their goals to achieve their objectives. That involves thinking of biorefineries as systems, from feedstock production to the replacement of a reference

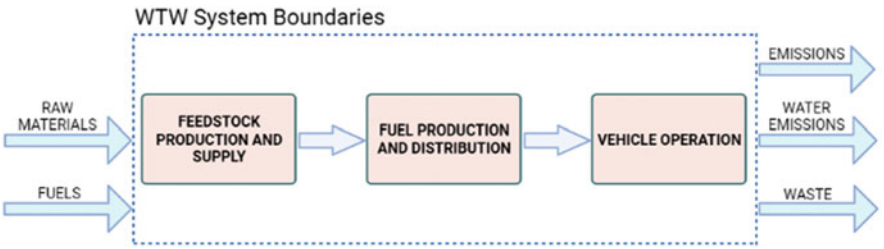

Fig. 8.5 The value chain for biorefineries, with the Well to Wheel (WTW) concept, which describes the idea of fuel production, processing, distribution, and use

product. The significant heterogeneity of the feedstock in biorefineries compared to petroleum refineries, and the substantial dependence of the biorefineries' products on feedstocks justifies their broader scope (Detroy 2018). Because of this significant reliance, the primary treatment of the feedstocks manufacturing, design, and engineering must be based on the product's requirement.

8.8 Challenges in Biorefineries

The concept of biorefineries is still in its early age (Rathore et al. 2016). The rapid depletion of fossil fuels and greenhouse gas emissions has caused a global crisis which has led to an increased focus on the development of technologies that are environmental-friendly, to produce energy, fuels, and other bio-based material products (Kapoor et al. 2017). The growing population of the world and its effects on world food security, as well as the emergency of mitigating climate change, are the points at issue that encourage the development of efficient ways to utilize natural resources, considering the social, political, and technological aspects, to produce bio-based products for human welfare (Cherubini et al. 2009). However, most of the recent research and investigation carried out for the development and optimization of biomass supply chains are focused on the economical aspect, while sidelining other critical aspects like sustainability, which amounts to a significant drawback in this field of work. One aspect that requires critical research and brainstorming is whether the modern biorefineries should be modeled towards the production of a completely new line of products, like fine chemicals that serve as precursors for the formation of high-value chemicals, or to produce raw materials that could serve as feedstock for the existing biorefineries and chemical plants (Lewandowski 2018). If this aspect is resolved, the issue of long-term sustainability will be sorted, and it will also be helpful in continuing the usage of the already available infrastructure network which took decades to reach where it is today.

Slow reaction rates are one of the main constraints for biological conversions in biorefinery processes, therefore increasing reaction rates is critical (Xu and Ferdosian 2017). Another limitation is that product concentrations are generally

low, resulting in high product recovery costs with current technology. In some multiple product systems, decreased yields of targeted products are common (Koyande et al. 2019). As a result, biorefinery technologies must be competitive and cost-effective to become a viable alternative to fossil fuels and petroleum-derived products. The geographical implications of biomass in connection to biorefineries also constitute a significant challenge that must be addressed in detail in future sustainability studies. In practice, elements such as sourcing, collection, transportation, and storage of input materials and by-products must be carefully examined because they have a direct impact on the long-term use of feedstocks (Wenger and Stern 2019). Further investigation into actual practice is highly recommended to fully realize the potential of biorefinery systems for a circular bioeconomy and see their promised effects in real-world scenarios.

There is still a significant gap between circularity expectations and biorefinery performance. The precise responsibilities of stakeholders—local and international government agencies, academia, and private firms—as well as their interrelationships, are yet unclear, necessitating systematic and extensive investigations. Recent scientific findings must be transmitted to the concerned private sectors, and policy frameworks and incentive structures must be linked with them (Bose et al. 2021b). They, in turn, initiate the implementation of these ideas into effective practice and provide timely feedback on potential areas for development.

8.9 Outlook and Future Perspectives

Successful implementation of the biorefinery concept helps in achieving broader objectives of clean technology and eco-friendly process. Modern integrated biorefineries are a key to building innovative biomass-based industries for clean and sustainable production of energy, fuel, chemicals, and other high-end products. Biomass wastes like manure and lignocellulose can be used which provides a cheap and renewable source of feedstock that brings about more environmental and economic benefits (Rezania et al. 2020). Figure 8.6 shows a similar concept of prospects for biorefineries.

Additionally, there is a huge potential for the growth of microalgal biorefineries where microalgae can be used as either a biochemical convertor or feedstock in the bioprocess (Arora et al. 2021; Goswami et al. 2022b, c). They have been recognized as one of the most attractive and sustainable feedstocks, but their use is limited by a lack of research and understanding of the commercialization of design and development. This is due to challenges in microalgal cultivation and supply, integration, and scalability of microalgal bioprocess and excessive water and energy needs that make their use costly. Moreover, the growth of microalgae is highly variable depending upon geographical conditions and environmental changes.

These limits in the same or other form are applicable to all the biorefineries (Koyande et al. 2019). Increasing environmental awareness and growing market share of biofuel production have put the use of the biorefinery concept in direct

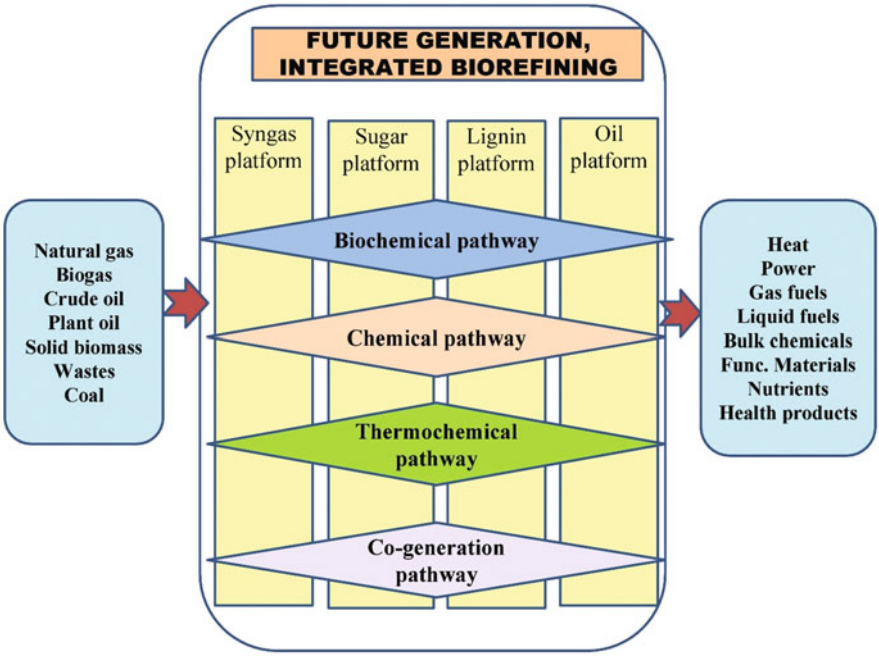

Fig. 8.6 Schematics for using fossil fuels along with waste biomass in biorefineries to develop functional bioproducts, following various established chemical and biochemical routes

competition with a petroleum refinery. Unlike conventional refinery, biorefinery faces several socio-economic and ecological challenges that have gravely impacted the efforts of sustainable production of energy and chemicals. These include aspects like biomass feedstock availability, storage and transportation, pretreatment, microbial development, yield, cost-effectiveness, and process development (Shylesh et al. 2017). All these aspects collectively contribute to the future commercial prospects of biorefinery.

To make biorefineries more competitive against petroleum refineries, economical production, greater yield, and product affordability are required. Significant research and investment by both public and private players are needed for proper optimization and integration of biorefineries in the manufacturing sector to replace traditional environment degrading industries. Moreover, the governments should enforce legislation and policies in favor of biorefineries to tackle major environmental complications caused by traditional industries. Biorefineries can lead forward the future of humanity and earth by becoming a key to fulfilling the sustainable development goals and environmental conservation targets and adding a strong concept bioeconomy into the economic landscape of global communities.

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

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Chapter 9
Biomass Recalcitrance and Omics Approaches for Understanding the Chemistry Affecting Recalcitrance

Surbhi Khanna, Ravindra Kumar, and Praveen Kumar Ghodke

Abstract Global population growth has increased the need for energy resources. The increased demand for energy has already begun to have negative consequences for the environment. This involves climate change and energy resource depletion, including fossil fuels. In order to avoid the exhaustion of such resources, fresh opportunities for the current scenario must be explored. Lignocellulosic Biomass (LB) is the easily available source form of renewable and sustainable energy. The LB consists majorly of cellulose, hemicelluloses, and lignin. Wheat straw, rice straw, sugarcane, and maize stover are the source of lignocellulosic biomass used for production of biofuels and high valued compounds such as ethanol, acids, and phenols. However, it is limited by recalcitrance phenomena which are subjected to various pretreatment methods leading to the production of biorefineries. Thus, this chapter will discuss methods in biomass pretreatment, factors contributing to recalcitrance, and the role of omics techniques in knowing the elements affecting recalcitrance.

Keywords Biomass conversion · Microorganisms · Bioenergy · Microfibrils · Renewable energy · Omics

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Abbreviations

AA	Auxiliary activity
CAZymes	Carbohydrate-active enzymes
GHs	Glycoside hydrolases
LB	Lignocellulosic biomass
LPMOs	Lytic polysaccharide monooxygenases
NGS	Next-generation sequencing
TF	Transcription factors

9.1 Introduction

The population growth is expected to grow exponentially with the increase in demand for energy. The statistics taken from a United Nations report named World Population prospects 2019 indicate an increase in the population of the world from 8.5 billion in 2030 to 9.7 billion by 2050 (United Nations (UN) (2019)). This expected increase in the population is an alarming situation, followed by energy resources depletion including fossil fuels. The negative consequences of it have already started showing their effects on the environment. As a result of which it becomes essential to explore new alternatives by which exhaustion of such energy resources can be reduced or limited. To this, biomass has opened up as one solution as it is a source of cheap, renewable, and sustainable energy. It includes agriculture residues such as wheat straw, rice straw, bagasse, sugarcane, maize stover; forest woody waste, municipal solid waste and referred as Lignocellulosic Biomass (LB) (Kumar et al. 2020). Thus, the use of such lignocellulosic biomass for the production of biofuels and high-value chemicals has importance for industries with the ability to provide energy sustainability without affecting the environment irrespective of the non-renewable resources (Patel et al. 2019).

9.1.1 Sources of Lignocellulosic Biomass

The sources for LB are easily obtainable and include the following:

(a) *Agriculture Residues:* The agricultural residues like rice stalk, wheat stalk, corn cobs, corn stover, corn stalk, and sugarcane bagasse have a harvesting time of short period because of which it can be available them all year round. The crops harvested annually are approximately 350–450 million tonnes, producing an abundant volume of these agricultural residues (Liyamen and Ricke 2012; Agrawal and Verma 2020).

(b) *Forest Woody Waste:* The forest woody waste includes the dried stems, pods, husks, tubers, legumes, and the mixture of all these which can be obtainable

during forest thinning and timber harvesting. Also, other forest residues like sawmills, wood chips, dead fonts, and switchgrass are known for generating high amounts of glucose and resistance against other diseases. This woody waste can be available at a low cost with high energy content (Zheng et al. 2014). Also, having a high lignin content, it produces less ash, making it one of the important biomass feedstock (Liyamen and Ricke 2012). Many plant species belong to the genus *Populus* and grass yield species.

(c) *Municipal Solid Waste*: This includes dried household waste and waste from food and pulp industries. The content of this type of source can be used for the production of ethanol and as a growth factor for the microbes involved during fermentation.

(d) *Microalgae:* It is a prominent source of biomass being a rich source of carbohydrate content (Cheah et al. 2016a, b; Goswami et al. 2022a, b). Also, it has high photosynthetic activity and biomass production yield (Cheah et al. 2015; Goswami et al. 2021a, b). The various microalgae species like *Spirulina sp*., *Spirogyra sp., Dunaliella sp.,* and *Chlamydomonas sp.* are the source of it.

9.2 Biomass and its Composition

For the conclusive production of biorefineries, the biomass composition is of very importance. The LB has the basic chemical composition of majorly three components: (1) hemicellulose; (2) cellulose; (3) lignin and minorly two, i.e., (4) volatiles and (5) ash. The list of lignocellulosic biomass compositions from several sources is shown below in Table 9.1.

Looking at cellulose with molecular name as $(C_6H_{10}O_5)_n$ is the linear polysaccharide consisting of K-glucose subunits having linkage with β-(1,4) glycosidic bonds. The reactive hydroxyl groups of its structure provide a property of diversification which can be derivatized into the material. Also, they have several other properties like biocompatibility, stereoregularity, and hydrophilicity.

The next major part of LB is hemicelluloses. It's an amorphous heteropolymer comprised of various polysaccharides such as xylan, galactomannan, glucuronoxylan, and xyloglucan having linkage of β-(1,4) or β-(1,3)-glycosidic

Table 9.1 Composition of lignocellulosic biomass from several sources

Source of lignocellulosic biomass	Cellulose (% value)	Hemicellulose (% value)	Lignin (% value)	References
Sugarcane bagasse	40–45	33–35	20–30	Cardona et al. (2010)
Corn cob	41	31	12	Chen et al. (2010)
Bamboo	45	24	20	Li et al. (2015)
Rice straw	38	32	12	Lu and Hsieh (2012)

Table 9.2 Percentage content view of lignocellulosic biomass

Composition	Weight ranges (%)
Cellulose	40–50
Hemicellulose	25–30
Lignin	15–20

bonds with each other (Zhou et al. 2017; Bhardwaj et al. 2021). It has many properties which make it an extensive choice for use in industrial applications like a strong binding agent with the presence of heterogeneous sugar groups and non-crystalline nature along with the low degree of polymerization.

The third part of LB is lignin composed of basic units like p-hydroxyphenyl, guaiacyl, and syringyl. It's a 3D cross-linked complex polymer structure consisting of phenyl propane structural units. Because of the presence of its complex array of aromatic alcohols and intertwining with the cellulose and hemicelluloses, it gives the property of rigidity to the lignocellulosic materials. The average percentage content view of LB is shown below in Table 9.2.

9.2.1 Importance of Biomass

The produced biofuels and chemicals totally depend on the lignocellulosic biomass material used. So, biomass selection is very important along with its structure in bringing out the good quality of lignocellulosic materials. For this, various important factors and parameters are associated which can affect the production of biofuels. Majorly contributing to it are the genetically controlled species variability, different harvesting conditions of source biomass, its production and storage practice, carbohydrate dispersal, ash and moisture content (Williams et al. 2017). Thus, the conversion of heat energy on burning of biomass into chemical energy is restored in the form of multiple units of polysaccharides as carbohydrates contribute to providing the end product ultimately.

9.3 Pretreatment Methods for Lignocellulosic Biomass

Lignocellulosic biomass has the nature of showing the recalcitrance phenomena. In general, the resistance of biomass structure undergoes the chemical and biochemical breakdown (Baruah et al. 2018; Bhardwaj et al. 2020a, b). This is a major challenge in the biorefinery process. To overcome this, various pretreatment technologies have devised that aid in the transformation of LB into the final bio-products. Pretreatment of biomass allows the easy accessibility of binding sites for enzymes and increases the surface area also (Parthiba Karthikeyan et al. 2018; Kumar and Verma 2020a, b). Different LB types have prerequisite pretreatments by which they can ferment the

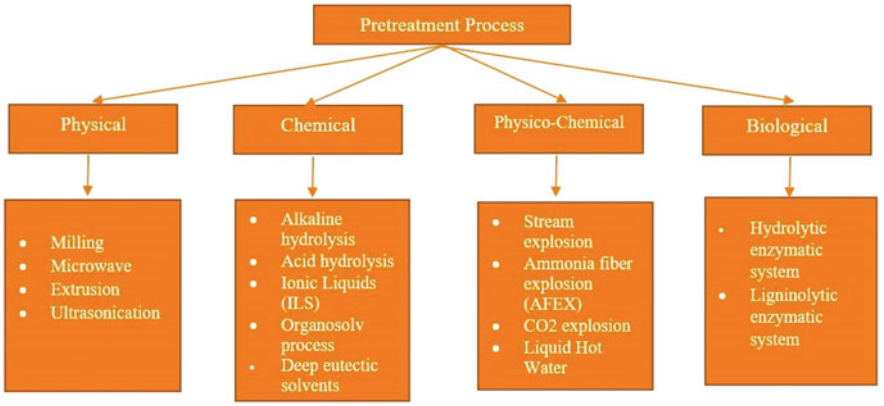

Fig. 9.1 Various pretreatment methods used for lignocellulosic feedstock

sugars easily. And for such results, every pretreatment applied to biomass should have the following characteristics:

- All types of lignocellulosic feedstock are treatable, for downstream processing sugar yields must be increased;
- Formation of co-products and inhibitors in less quantity;
- Generation of pretreatment methods produces low energy and cost (Prasad et al. 2016; Kumar and Sharma 2017).

A pretreatment method to be chosen depends upon the biomass selected and the environmental factors associated with it. This is because of the variation in constitution percentage for each different source of biomass. Pretreatment processes are of different types and include

- Physical pretreatment method
- Chemical pretreatment method
- Physicochemical pretreatment method
- Biological pretreatment method

The various pretreatment methods used for biomass are shown below in Fig. 9.1.

9.3.1 Physical Pretreatment Method

The physical pretreatment method is required for every other pretreatment method to obtain increased surface area, decreased polymerization, and crystallinity by the reduction in the particle size (Rajendran et al. 2017; Verma 2022). This method is found to be environmentally friendly and rarely produces toxic chemicals (Shirkavand et al. 2016). On the contrary, disadvantages of these methods are high

energy consumption and the cost of mechanical equipment. Thus, there are the following methods under the physical pretreatment and these are the following:

9.3.1.1 Milling

It is widely used for pretreating biomass as it can reduce the size of particles by 0.2 mm (Baruah et al. 2018). The parameters that are to be considered for efficient milling procedures are the initial biomass size, machinery parameters, feeding rate of biomass, time, and moisture content (Amin et al. 2017; Jędrzejczyk et al. 2019). Related to *ball milling methods*, the studies have found the effect of pretreatment on pre-milled wood fiber with enhanced energy consumption along with a high yield of glucose and enzymatic hydrolysis procedure (Gu et al. 2018). Another research study (Phanthong et al. 2016) has indicated that using this method on cellulose paper and powder gives high production of nano-cellulose along with high thermal stability and crystallinity.

9.3.1.2 Microwave

In this pretreatment method, there occurs polarization which causes molecular collisions leading to the generation of thermal energy causing the disarrangement of biomass (Aguilar-Reynosa et al. 2017). The advantages of this pretreatment method are energy efficiency, producing very low inhibitors, easy to perform, and within a short interval of time giving high heating capacity making it convenient to go for (Tayyab et al. 2018). In addition to this, the microwave method has been analyzed and studied in combination with other pretreatments for the upliftment of the process. In the species of *Hyacinthus,* microwave pretreatment was given and studied for the increase in the quantity of methane gas under anaerobic conditions. The results obtained with it show the yield of 221 mL·g-sub^{-1} of methane which is high in number and a percentage value (Zhao et al. 2017).

Study of microwave pretreatment method is frequently done from a delignified hardwood kraft pulp along with alkaline which allows segregation (Liu et al. 2018). On applying the microwave treatment, the structure was disrupted efficiently in comparison to the alkaline chemical method. The outcome of it shows the extraction of LB substrates, i.e., 93.05%.

9.3.1.3 Extrusion

In the extrusion pretreatment method under controlled temperature and increased pressure ($<300\ ^{\circ}C$) conditions, the biomass is passed through a tight barrel where its structure of it gets disrupted. This is because of the pressure of shear forces and high temperature on the revolving screws of the barrel (Kumar and Sharma 2017). The key features of this method making it efficient for use are discussed. This

pretreatment method is executed by using different additives, and various parameters such as screw speed, design, and temperature of the barrel are important (Duque et al. 2017).

Taking into account these above-mentioned factors, research studies are designed where only the extrusion method is applied and the experimental outcome of it has shown the increased yields of glucose apart from the application of combined pretreatment methods. An example of it is the use of bagasse and glycerol; straw and glycerol have a ratio of 1:0.75 and 1:0.5, respectively. The pretreatment applied here is only the extrusion method producing a yield of glucose on enzymatic hydrolysis with a percentage factor of 68.2%, apart from the pretreatment on olive tree pruning in which alkaline treatment is combined with extrusion method, glucose yielding is 69% (Negro et al. 2015).

9.3.1.4 Ultrasonication

The ultrasonication pretreatment method is performed using ultrasonic radiations by following the principle of cavitation. This treatment provides less processing time, low temperature, and less chemical consumption. It carries out the extraction of lignocellulosic substrates by producing shear forces that breaks the interlinked structure of subjected biomass (Ravindran and Jaiswal 2016). The various factors such as its frequency, duration, power, and temperature are important for the regulation of ultrasonication pretreatment. Some of the research studies on this method along with their results are discussed below:

In addition to it, ultrasonication pretreatment was performed on sugar beet shreds. After enzymatic hydrolysis, the yield of cellulose is 3.7 times more than the untreated samples (Ivetić et al. 2017). In the same year, interesting work is represented (Luzzi et al. 2017) in which the ultrasonic pretreatment was found to be very efficient as applied alone along with enzymatic hydrolysis applied in a one-step. The results indicated that there was a yield of 15.5 UPF mL^{-1} by enzyme cellulase at a controlled temperature of 40 °C and pH 4.6 and applied power at 44 W. Thus, it can be concluded from the above-mentioned research study that the ultrasonication pretreatment method can be employed for biomass disruption efficiently.

9.3.1.5 Freezing

This pretreatment method for biomass is based on volumetric changes in water. With the change in water state, i.e., liquid to solid state at low temperature, there is a coincided change in volume of water which results in cell wall breakdown (Rooni et al. 2017). Apart from other discussed physical methods where inhibitors are formed during pretreatment, freezing has no such problem. In addition, this method is low in cost and eco-friendly (Cheah et al. 2020). Studies have found that performing the freezing method has resulted in a good amount of glucose yield.

9.3.2 Chemical Pretreatment

Under this, five different methods are presently being used for pretreatment and are discussed below here.

9.3.2.1 Alkali Pretreatment

This pretreatment is important with its effective use. The basic principle involved in this is the cellulase delignification by use of potassium hydroxide, sodium, calcium, and ammonium. This delignification occurs by swelling in cellulose leading to a change in the structure of biomass under treatment along with crystallinity. This causes an increase in the surface area of the subjected biomass structure (Behera et al. 2014). With the lignin during alkali pretreatment, accessibility of hemicelluloses to carbohydrates for enzymatic hydrolysis improves by elimination and substitution of acetyl groups by uronic acid, respectively (Maurya et al. 2015). The research studies on alkali chemical pretreatment show its advantages of it.

The alkali pretreatment was used in sugarcane bagasse with lime and alkaline peroxide producing a high yield of 200 mg g^{-1} with lime of content 0.04 g g^{-1} for 37 h at 70 °C (Rabelo et al. 2011). This shows the suitability of treatment for the biomass. In 2016, Talha et al., for increasing the production of biomethane, made the conditions of alkali treatment optimized. This shows the result of an increase in methane by 88.20 wt% and elimination of lignin at 86.27 wt% with the use of 1% NaOH alkali for 3 h at 100 °C.

9.3.2.2 Acid Treatment

Acid pretreatment works because of the sensitivity of glycosidic bonds to the action of acid which is present between the hemicelluloses and cellulose in lignocellulosic biomass structure. The acid-catalyzed reaction produces H^+ ions which break the bonding between cellulose and hemicelluloses. The acid pretreatment is divided into categories for its usage, concentrated acids, and dilute acids. The concentrated acids can be used for acidic pretreatment at 30–70% concentrations with low temperatures (<100 °C). These can cause the conversion of sugars at a speedy rate. This is because of the corrosive and toxic nature of concentrated acids. Whereas the diluted acids can be used at 0.1–10% concentration at high temperatures (100–250 °C) (Baruah et al. 2018). Besides this, there are disadvantages associated with acidic pretreatment which include the generation of inhibition which needs a substantial amount of washing before the fermentation process, and the corroding of reactors because of the corrosive nature of concentrated acids (Kumar and Sharma 2017). In relation to this, research studies have been done indicating the advantages of pretreatment with dilute acids also.

Bambusa spp. varieties are pretreated with dilute acid and studied for bioethanol production. The outcomes of it have indicated that dilute sulfuric acid is shown to be the best pretreatment agent (Sindhu et al. 2014). Also, a study on wild rice grass (*Zizania latifolia*) while studying the comparative effect of dilute acid with that of alkali was done. The results have shown that treating with 0.4% H_2SO_4 with 10% biomass loading releases 163 mg sugar g^{-1} while only 92 mg sugar g^{-1} biomass was obtained when treated with 1% NaOH (Sahoo et al. 2018).

9.3.2.3 Ionic Liquids

This pretreatment method is extensively a new approach using ionic solvents (cations and anions) with a melting point <100 °C. Cations and anions are used as cellulose solvents, i.e., take part and perform the solubilization of cellulose and lignin. These ionic solvents have various advantages such as chemical stability, non-toxicity, and adjusting the nature of cations and anions making it the specific property on which the principle of pretreatment depends (Chen et al. 2017). For example, imidazolium-based ([(C3N2)Xn]+), pyridinium-based ([(C5N)Xn]+), pyrrolidinium- based ([C4N)Xn]+.

Studies done associated with ionic liquid solvents have demonstrated their advantageous part. Pretreatment with a series of pyrrolidinium-based ionic solvents on corn stalk is performed at 90 °C for a time of 30 min. The observation of such comparison gas showed the elimination of 85.94% lignin which decreased sugar yield (Ma et al. 2016). On pretreatment of rye straw using 1-ethyl-3methylimidazonium with optimum temperature and time, a three-fold increase of the reduced sugars yield rather than with untreated one was observed. Thus, showing the strong impact of pretreatment on the biomass (Smuga-Kogut et al. 2017).

9.3.2.4 Organosolv Process

In the organosolv pretreatment method, organic solvents are used for breaking down the internal bonding between hemicellulose and lignin, the rest remaining with cellulose. The basic principle of this type of pretreatment is the solubilization of hemicellulose and delignification that occurs leading to an increase in pore volume and surface area of cellulose by enhancing the accessibility of enzymatic hydrolysis and saccharification (Zhang et al. 2016). For example, acetone, methanol, ethanol, and ethylene glycol. With this treatment too, advantages and disadvantages are present. Advantages include easy recovery and the use of lignin obtained after pretreatment for industrial applications whereas disadvantage includes the high cost of organic solvents.

9.3.2.5 Deep Eutectic Solvent

This pretreatment method uses solvents at temperatures <100 °C. They consist of ionic fluids with two or three components, interconnected with H-bonds leading to the formation of a eutectic mixture with a melting point lower in temperature than each component. The major advantage of it compared to ionic solvents is that they are environmentally safe and low cost (Zdanowicz et al. 2018). Research studies done regarding the pretreatment of deep eutectic solvents have found that the elimination of lignin and hemicelluloses is supported by the presence of acidic H donors and these solvents more efficiently (Xu et al. 2016).

9.3.3 Physicochemical Pretreatment

The methods that come under the physicochemical pretreatment use combined approaches, i.e., physical having use of mechanical force and chemical having use of chemical effects. The detailed description of these methods is as follows:

9.3.3.1 Steam Explosion

This method works by applying saturated high-pressure steam of 0.69–4.83 MPa at a temperature of 160–260 °C for the infusion of water molecules in the substrate structure. With the subsequent decline in pressure, the water molecules escape in an explosive way. The decline in pressure from high to low levels causes the biomass subjected to pretreatment to fulminate into split fibers (Baruah et al. 2018).

This method has many advantages like limited chemical usage and minimum environmental hazards, and the most important is the full fermented sugar retrieval (Pielhop et al. 2016). Also, it has been found that by the use of catalysts such as H_2SO_4, H_3PO_4, SO_2, or CO_2, the regulation of this method can be enhanced. The use of catalyst brings down the temperature and the residence time along with the generation of inhibitory compounds at a minimum level by enhancing the enzymatic hydrolysis with total hemicelluloses retrieval (Negro et al. 2014; Neves et al. 2016).

9.3.3.2 Ammonia Fiber Explosion (AFEX)

In this pretreatment method, a high temperature, i.e., 60–100 °C for 5–30 min is given to the subjected biomass in a closed vessel with a liquid state of ammonia in a 1:1 ratio followed by the rapid release of pressure (Shirkavand et al. 2016). With this, the following changes occur, i.e., elevated temperature and pressure cause swelling in the biomass, and rapid release of pressure disrupts the exposed biomass which

brings down the degree of crystallinity and increases the accessibility of enzymes (Baruah et al. 2018).

This method also has some keynote features like the formation of an insignificant number of inhibitors in comparison to other methods. The efficiency of this method for treating biomass has been studied by comparing the corn stover pretreated with AFEX alone and that with dilute acid. The results of it show that corn stover pretreated with AFEX is worthier than that pretreated with dilute acid (Mathew et al. 2016).

9.3.3.3 CO_2 Explosion

This method is used for the pretreatment of LB by using supercritical CO_2 at high pressure which causes CO_2 molecules to enter the subjected biomass. As pressure goes up, the rate of CO_2 by which it moves inside the biomass increases in cellulosic pores size. After getting dissolved with water, it forms carbonic acid causing the release of hemicellulose during hydrolysis with increased yield. On pretreatment of corn stover with the pressure of 24 MPa at 150 °C for 60 min with this method, a 2.5-fold increase in yield is observed in comparison to untreated corn stover (Narayanaswamy et al. 2011). Thus, this method has advantages like low cost of CO_2, non-toxic in nature, and most importantly reducing the greenhouse effect due to utilization of CO_2 makes itself an appropriate choice for pretreatment of LB.

9.3.3.4 Liquid Hot Water (LHW)

In this method, at high temperature and pressure, i.e., at 170–230 °C and up to 5 MPa, respectively, water is used. This high-temperature water makes the cellulose fibers open up by hydrolyzing the hemicelluloses with the liberation of acetyl groups and making the lignin detached from it (Zhuang et al. 2016). The increase in the yield of glucose with this pretreatment process has been proved. During the pretreatment of rice straw with liquid hot water, the use of alkali and acid as promoters are studied and it has been found in making changes in the structure of LB (Imman et al. 2014). Thus, can be used for the industrial application in respect of costs, as it does not require chemical and catalyst, no inhibitor formation, no size reduction requirement prior to pretreatment, and has the ability to pretreat up to 80% of hemicellulose (Menon and Rao 2012; Kim 2018).

9.3.4 Biological Pretreatment

In this pretreatment method, naturally occurring microorganisms alter the biomass structure and breakdown into simple substrates for enzymatic digestion. The enzymatic digestion in microorganisms occurs through two systems (Waghmare et al.

2018; Alam et al. 2021; Agrawal and Verma 2021). Either by the hydrolytic enzymatic system or by the ligninolytic enzymatic system. In the second system, it disrupts the lignin of exposed biomass with the aid of degrading microorganisms such as white-rot and brown-rot in presence of enzymes named lignin peroxidase, versatile peroxidase, and manganese peroxidase.

Apart from this, other microorganisms like insects (Varelas and Langton 2017), gastropods (Trincone 2018), and worms ((Devi et al. 2019) can be used for this method. These groups of organisms have inbuilt physiological functional and enzymatic properties for the breakdown of cellulosic biomass. The microorganism has such microbiota capable of digesting carbohydrates, chitin, starch, and lignin. Thus, in order to regulate this pretreatment method in a better way, certain characteristics of these microbes are to be considered like aeration, temperature, moisture, incubation time, accessible surface area, species, interaction level, the role of enzymes and hydrolysate, composition of culture media, pH, C & N source and cellulose crystallinity, etc.

Therefore, both advantages and disadvantages of biological pretreatment exist. Disadvantages include its monitoring and observation of growth control for microorganisms. Thus, not suitable currently for large-scale applications. Apart from it has advantages like low energy consumption, no occurrence of chemical formation and eco-friendly (Cheah et al. 2020).

9.4 Biomass Recalcitrance and Bioconversion

Recalcitrance is generally meaning the resistance to a chemical decomposition or decomposing occurring at an extremely slow rate. Here, recalcitrance is associated with biomass. The plant cell walls of the LB emerged with the property which makes its cell wall difficult to deconstruct ultimately. This type of resistance of plant cells for the deconstruction of polysaccharide sugar polymers into monomeric sugars can be defined as biomass recalcitrance (DeMartini et al. 2013).

Biomass recalcitrance is the main blockade for the conversion of the cell wall polymers into reactive intermediates. Thus, it has emerged as a potential barrier that needs to be removed because, in comparison to the crude oil, the polysaccharide glucose units and aromatic alkyl polymers have higher oxygen contents. Reducing these high-density energy molecules into biofuels is a difficult task (Chundawat et al. 2011).

9.4.1 Factors Contributing to Biomass Recalcitrance

The understanding of the LB composition and structure can help in understanding its conversion of it into biofuels and high-value chemicals. The lignocellulosic substrate possesses the property of a plant cell wall which resists the breakdown of its sugar

components. It includes the natural, chemical, and structural means. These mechanisms are studied and shared in the technical report of the national renewable energy laboratory and it includes:

1. A dense mass of cells that forms the rind of grasses and bark of trees;
2. The cell wall of plants has complex nature of arrangement that counters the aqueous penetration from cell to cell;
3. The restricted liquid penetration of the vascular structures of the plant throughout the stem;
4. Crystalline nature of celluloses;
5. The instinctive property of enzymes while acting on insoluble surfaces like cellulose (Himmel et al. 2005).

Apart from this, it has some chemical and mechanical factors which bring down the enzymatic degradation of LB. This includes the mechanical pressure provided by plug feeders which may collapse the natural vascular system; petrification of cellulose with the microfibers, lamination of lignin on cellulose surfaces during the cooling down period after applying pretreatment approaches. The third point for understanding the biological bases or the structural aspects responsible for biomass recalcitrance includes:

9.4.1.1 Celluloses

In providing recalcitrance to the LB, the structure of the cellulose contributes. This includes the microfibril location, arrangement, and presence of the number of glucan chains in comparison to the proportion of crystalline core. For finding the nature of cellulose being crystalline or not, many studies have been done. So far. In a study with solid-state NMR data, it has been found that not all cellulose is crystalline. The surface glucan chains in it acquire different conformations in order to fit the amorphous chains other than the crystalline core (Viëtor et al. 2002).

Later on, the studies done on finding the nature of cellulose with solid-state NMR data have indicated the glucan chains which are insoluble to water making it crystalline. The diameters of cellulose microfibrils obtained from several plant species range from 2.3 to 3.0 nm corresponding to the glucan chains which are 18–24 in number (Fernandes et al. 2011; Newman et al. 2013; Thomas et al. 2013). Another analysis with wide-angle X-ray scattering is done, which has shown the involvement of glucan chains in providing crystalline nature to cellulose with a diameter of 3.3–3.6 nm (Liu et al. 2013; Thomas et al. 2014).

In getting more understanding of the microfibril arrangement in the cellulose, in wood microfibrils, the diameter is found to be variable ranging from 14 to 23 nm (Donaldson 2007). In 2012, Ding et al. studied elementary fibrils of maize stem cells with measurements of about 3×5 nm and reported that five of these fibrils unite into the microfibrils of over 20 nm. Hence, if we are able to know about the inter-microfibril interactions, then we can disarray the arrangement in order to easily go for bioconversion using reagents.

9.4.1.2 Hemicelluloses

As hemicelluloses provide properties of strong binding agents, it includes the various targets of inherent control such as polymerization, pattern substitution, and cross-linking of imitation sites. Considering polymerization, short-chain lengths and mutations are key players. And thus, these mutations related to it terminates the synthesis of the tetrasaccharide which distributes the length of polymer extensively. In contrast, the disrupted polymerization produces short-length polymers having extreme tetrasaccharides (Peña et al. 2007).

The property of substitution in hemicelluloses affects the binding with cellulose. The GlcA and acetate group substitutions degree and state on xylans can modify the binding ability. In *Arabidopsis*, the locked action of two enzymes named GUX1 and GUX2 is found by adding the (Me-) GlcA residues. The role of GUX enzymes is found in supporting and providing steric binding to cellulose microfibrils. The research done by Bromley et al. 2013 has indicated that in single mutants when GH30 glucuronoxylanase C family and the enzyme requiring a GlcA or Me-GlcA side-group for its activity were applied then GUX1 adds the substituents evenly paced xylosyl residues. Whereas the GUX2 is involved in placing GlcA or Me-GlcA residues mostly at fifth to sixth xylosyl residues. In the year 2014, Busse et al., supporting this property of hemicelluloses, predicted evenly spaced acetylation patterns which change the xylan backbone conformation from three-fold to a two-fold symmetry suggesting that it affects the solubility of xylan leading to changes in molecular interactions.

9.4.1.3 Lignin

This is the third major component of biomass that contributes to the total of 90% of LB. Since lignin is associated with providing rigidity to the biomass structure, its separation is very important because it causes the changes in basic lignin structure. Because of this, these structures are more arduous for handling its separation making the necessity to look for the genetic and other factors contributing to this property of lignin. In the lignin biosynthetic pathway, changes in the expression level of genes can drift the carbon flux from phenylalanine to S or G monolignols. With this, the expression of ferulate-5-hydroxylase (F5H) gene produces lignin, i.e., substantially the S-lignin (Stewart et al. 2009). This S-lignin is found to intensify the sugar yield under the enzymatic hydrolysis of cellulose (Li et al. 2010) and contribute to higher-order structural changes affecting cell–cell adhesion.

Yang et al. (2013) have done genetic engineering in *Arabidopsis* which is having high carbohydrate and low lignin content beyond compromising with its growth have changes in promoter sequences associated with transcription factors such as NAC or MYB DNA-binding domains. These transcription factors help in regulating the synthesis of individual cell components. Hence, identification and understanding

of different sources responsible for recalcitrance are still to be explored by looking at the genetic level of the biomass originating from different plants.

9.4.2 Biomass Conversion

Understanding the recalcitrance associated with LB is associated with biomass conversion. It can be divided into two parts, i.e., thermochemical conversion and or biochemical conversion.

9.4.2.1 Thermochemical Conversion

Under the thermochemical conversion, the four processes are included:

1. *Direct Combustion*
   Combustion involves the direct heating or burning of the biomass to produce heat ideally in a closed vessel or boiler usually at a temperature of 800–1000 °C resulting in conversion into CO_2 and H_2O (Sarkar and Praveen 2017). Oxygen required for this process is provided by bonded oxygen and rest from the air injection into the system.
2. *Gasification*
   Gasification involves the change in solid biomass fuel using gasifying agents such as steam, air, or CO_2 forming the combustible gases mixture like CO, CO_2, and CH_4 at a temperature of 800–1300 °C. The performance of a gasifier is basically dependent on the moisture content in the biomass feedstock. As the moisture in biomass increases, the efficiency of biomass conversion decreases including the production rate (Mandapati and Ghodke 2020).
3. *Pyrolysis*
   Pyrolysis is an exothermic anaerobic reaction where the breakdown of organic matter occurs in the presence of nitrogen. The biomass is heated in an inert atmosphere producing vapors and carbon-rich residues with energy requirements in the range of 207 and 434 kJ/kg. Thus, the vapors produced contain cellulose, hemicellulose, and lignin polymers while the remaining carbon residues are leftover biochar (Ghodke and Mandapati 2019). Factors like environment, catalysts, pressure, heating rate, temperature, residence time, etc. play an important role in this process.
4. *Torrefaction*
   In this conversion process, the main target is to increase the biomass energy density along with the product's properties, i.e., fuel (Ghodke et al. 2021). It is effectuated at a high temperature in which the components of biomass partly decompose. The final product procured at the terminal stage is a dry and black residual solid named terrified biomass.

9.4.2.2 Biochemical Conversion

Under the biochemical conversion, the biomass is converted using the biological pretreatments by means of microorganisms. The use of biological pretreatments provides the platform for obtaining the fuels and chemicals such as hydrogen, ethanol, biogas, and organic acids (Chen et al. 2010). The main advantages of this conversion process are purity, efficiency, and clarity (Chen et al. 2017). The processes that come under it are:

1. *Anaerobic Digestion*
   It includes the anaerobic digestion of LB to produce bioenergy. It is very sustainable and cost-efficient for bioenergy production. This conversion process minimizes the amount of waste but not the bioenergy. The anaerobic digestion of LB produces the biogas, i.e., methane (CH_4). This process is carried out at a temperature range of 30 and 35 °C, or 50 and 55 °C using two stages. In stage first, the complex organic biomass breaks into simple compounds (such as acetic acid and propionic acid) followed by the second stage of conversion into biogas along with carbon dioxide. The biogas, methane is produced with the aid of methane-producing bacteria.
2. *Fermentation*
   It is the simple process in which the enzymes secreted from the microbes change the fermented product, i.e., sugars into alcohols and acids. The extent of the fermentation process depends on the nature of biomass. As it can lead to excess sugar formation in comparison to polymeric substances. Therefore, to overcome this microbial metabolism, degradation and saccharification of the biomass cell wall are considered (Zhang et al. 2010; Cheng et al. 2012).

9.5 Role of Omics Approach in Understanding the Biomass Structure and Chemistry Affecting Recalcitrance During Pretreatment

After getting an understanding of the biomass and the various factors associated with it, providing biorefineries is the major concern to improve product value. This can be done by further refinement of pretreatment methods for LB. And by looking into the present scenario, the role of "OMIC" approaches is of major importance. The scope of novel research which could be cost-effective and fast for biomass pretreatment methodology followed by its conversion into a beneficial biorefinery is being explored by applying omic approaches like genomics, proteomics, transcriptomics, and next-generation sequencing (NGS). This could help study the factors affecting biomass recalcitrance.

With this advent of biological pretreatment methods that include the microbes as well enzymes along with the combination of different omics approaches, the scope

of research has enlarged. For reducing this recalcitrance, the various approaches of omics are used in the past and are still being used.

9.5.1 Transcription Factors and Genomic Tools

The transcription factor (TF) mediated control has been found to be the important one so far. The quality of biomass gets affected by changes in the cell wall properties. For the formation of secondary cell walls, TFs are found to be involved in the regulation of lignin biosynthesis (Kalluri et al. 2014). In *Arabidopsis,* the presence of master switches that act as regulators of recalcitrance-related properties is found. They regulate the genes encoding other down streaming TFs. In AP2/EREBP, the transcriptional factor (SHINE2) has been a key regulator and is identified for increasing and decreasing the cellulose and lignin biosynthesis, respectively (Aharoni et al. 2004; Ambavaram et al. 2011). Thus, misexpression of these TFs has shown short stature, weak walls, or imbalance in other wall chemicals, proposing a strong and efficient systems biology model of cell wall changes and a strategy for achieving the desired wall property precisely (Kalluri et al. 2014). Resources for understanding the plant cell wall formation and other factors with help of omics are given below:

For in situ cell wall measurements, phenotyping techniques include label-free methods of coherent Raman scattering microscopy. For the study of in situ cellulose biosynthesis and cellulose degradation related investigations fluorescence tag-based single-molecule tracking of protein (Gu et al. 2010) and imaging of fluorescent lignin monomers in cell walls is present (Ding et al. 2012; Tobimatsu et al. 2013). Annotated genome sequences from dozens of plant species, hundreds of genotypes, standards-adhered data analysis, and reconstructions of transcriptional networks (Kalluri et al. 2014).

9.5.2 Transcriptomic and Proteomic Approach

The wood decay basidiomycetes, e.g., Brown rots and white rots are important and specialized in the degradation of recalcitrant biomass, by having the presence of carbohydrate-active enzymes (CAZymes) for the production of value-added bio compounds. In regard to this, *Laetiporus sulphureus* ATCC 52600 fungus has been studied by using a multi-omics approach (de Figueiredo et al. 2021). The details and key findings of this research are as follows:

L. sulphureus ATCC 52600 genome was sequenced, and phylogenomic analysis is done followed by growth analysis. The outcome indicates flexibility of its metabolism with mono and disaccharides along with polysaccharides from biomass. The transcriptomic and proteomic profile for *L. sulphureus* was constructed and analyzed. The results indicate the presence of upregulated transcripts and CAZymes.

Cellulase and hemicellulose are found along with 112 proteins making it to conclude the presence of oxidative and hydrolytic metabolism for the recalcitrant biomass.

9.5.3 Metagenomic Analysis

Metagenomic analysis was performed on the soil microbiomes using samples taken from 5 different locations in China. For analyzing the microbiomes, the strategy was constructed, and the cellulolytic microbial consortia are named FIRT. On analyzing the FIRT, 7 individual phylotypes are found and subjected to reconstructed bacterial draft genome analysis. The results demonstrated the presence of cellulose-degrading capability naturally in the seven found microbial phylotypes. (Zhou et al. 2014).

9.5.4 Secretomics

Using the proteomics and tools associated with it, enzymes responsible for biomass disruption can be identified, and the study of these secretions of protein is of very importance. An example of it is the glycoside hydrolases (GHs). These enzymes are either lignocellulolytic bacteria or fungus, for example, *T. reesei.* These microbes can be stimulated for the production of such enzymes by providing suitable growth medium conditions (Sethupathy et al. 2021).

Next to GHs are the auxiliary activity (AA) and lytic polysaccharide monooxygenases (LPMOs). They show involvement in biomass hydrolysis using oxidative cleavage for breaking glycosidic linkages, e.g., AA9 which is an LPMO. Its main function is to break cellulose and make it under stress to lose its strength. Also, ligninolytic enzymes can contribute to reducing recalcitrance by screening under the secretomes approach. For example, secretome analysis of *Fusarium solani* MYA 4552 grown on a medium rich in lignin reveals the presence of proteins like laccases, oxidoreductases, dioxygenase, and catalase. Hence, the use of the secretomes approach provides low cost and high biomass utilization efficiency.

9.5.5 Next-Generation Sequencing

This sequencing technology has shown promising opportunities. For plants like sugarcane, the whole genome sequencing was made possible, for e.g., cultivar R570 and tetraploid *S. spontaneum* genotype (AP85-441). Being cost-effective, it has let the researchers look and study for molecular markers that can help in breeding. The technology has given aid by identifying various alleles of the same genes. This application is yet to be explored for other sources of LB, i.e., except

sugarcane for understanding the recalcitrance of biomass from data provided by NGS.

Hence, the omics approach has arrived as an inventive field of research that can be used further for understanding more about the biomass recalcitrance phenomena and its other associated factors which ultimately leads to the increase in biofuel and biochemical production efficiency.

9.6 Conclusion and Future Prospective

With the exhaustion of reserves and ruthless burning of fossil fuels, research is moving towards the exploration of new energy resources that are replenishable. For this, LB has evolved as a prospective candidate for a renewable source of energy. In the present scenario, LB has proven itself as a perfect switch for fossil fuels. But the recalcitrance property exhibited by the biomass is still an issue that needs to be completely resolved as result, the full utilization of biomass is not achieved. Though present pretreatment methods have their advantages and disadvantages. However, advancements in omics have opened perspectives to develop future energy crops resulting in the replacement of fossil fuels with biofuels.

But still, there is no independent technology available that can undergo a conversion of LB into bioenergy. Also, the need is there to look out for novel pretreatment methods as it is becoming a major challenge for biomass processing industries.

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

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Chapter 10
Demonstration of Application of Fungal Xylanase in Fruit Juice and Paper Deinking and Validation of Its Mechanism Via In Silico Investigation

Mamta Yadav, Komal Agrawal, Bikash Kumar, and Pradeep Verma

Abstract India is the leading provider of agricultural and forestry-based sustainable resources, that are typically considered waste, that is why developing a low-cost method for xylanase production holds great promise for converting discarded and underutilized lignocellulosic biomass (LCB) into usable products. In-house fungal cultures of *Rhizomucor variabilis* (Ac. No. KC602326.1) and *Penicillium* sp., (Ac. No. FJ430745.1) produced 18.3 IU/mL and 66.5 IU/mL of xylanase using rice straw as a carbon source. Furthermore, xylanase showed promising potential for use in the clarification of fruit juice as the enzyme was proved to be efficient in increasing reducing sugar, filterability, and clarity (%) from fruit juices. The paper industry has long been known as one of the major reasons for environmental pollution due to its high energy and chemical usage. The use of xylanases in the paper industry has been correlated to less pollution caused by the use of large amounts of chlorine and chlorinated compounds during pulp bleaching. Because of the depletion of fossil fuels, there is an urgent need to develop eco-friendly and long-term energy sources. As a result, the use of low-cost lignocellulosic materials is paramount for the cost-effective production of xylanase. In silico tools were also used to compare the results of wet lab experiments by docking xylanase with the ligands xylan, cellobiose, and lignin. In silico analysis of xylanase from the model fungal organisms had the highest affinity for xylan followed by cellobiose and

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lignin. Thus, it has future potential applications in the fruit juice clarification and paper and pulp industry.

Keywords Fungi · Xylanase · Juice, paper, and pulp · In silico

Abbreviations

BLAST	Basic Local Alignment Search Tool
DNSA	3, 5-dinitrosalicylic acid
FASTA	Fast-All
GHG	Greenhouse gases emissions
LCB	Lignocellulosic biomass
LPCB	Lactophenol cotton blue
NCBI	National Center for Biotechnology Information
PDA	Potato Dextrose Agar
PDB	Protein Data Bank
SMR	Swiss Model Repository
XOS	Xylooligosaccharides

10.1 Introduction

Expanded urbanization, industrial, and agricultural wastes have enhanced the emission of greenhouse gases (GHGs) and the depletion of fossil fuels (Kumar and Verma 2021a). As, a result of which the use of agricultural residues can be a potential and renewable resource to substitute fossil fuel (Bhardwaj et al. 2021). The concern with climate and sustainability has been rising logically, and sustainable resources of materials have been progressively explored (e.g., lignocellulosic biomass—LCB) (Bhardwaj et al. 2020; Kumar and Verma 2021b). A huge amount of LCB such as wheat straw, rice straw, rice husk, and bagasse are generated in large amounts annually throughout the globe and its disposal creates various problems. The farming build-ups left after collecting are either scorched nearby (which makes air contamination and impacts soil quality) or put away as stores in the field (Kumar and Verma 2020). As a result, agricultural residue management is becoming one of the most pressing issues, and management of leftovers can help to deal with and accomplish low carbon dioxide emanations and the generation of biofuels or value-added products (Adejumo and Adebiyi 2020). Solid waste can be used as a probable source for mass production of a variety of industrially valuable products such as enzymes, chemicals, biofuel, and animal feed (Abdel-Shafy and Mansour 2018). The use of LCB as feedstock in various bioprocesses not only offers a substitute for low-cost substrate but is also a good strategy for reducing pollution problems induced by their frequent inappropriate release to the environment (Singhvi and

Gokhale 2019). As a result, there has been a growing interest in using agricultural residues which are considered to be waste and cause environmental pollution, and require a high amount of expenditure for proper management (Kumar and Verma 2020).

Agricultural leftover biomass is rich in lignocellulose and predominantly contains lignin, cellulose, and hemicellulose. LCB is considered as most abundant renewable biomass on earth. Lignocellulose-based wastes are collected per annum in huge amounts, causing ecological issues. However, several reported literature showed that LCB has sugars and other valuable biomolecules which can be used for the production of various bioactive products such as enzymes, bioethanol, organic acids, food additives, etc. (Kumar and Verma 2019). The utilization of LCB provides numerous bioproducts simultaneously it also reduces the pollutant from the environment which arises due to the accumulation on the earth (Bhardwaj et al. 2021).

10.1.1 Molecular Docking

Molecular docking is the process where a ligand docked to the active site of a receptor and then searching for an appropriate alignment and configuration to achieve the best match of shape and interaction between the receptor and ligand (Jin-Xia et al. 2007). It is a structure-based drug design approach that imitates molecular interactions and anticipates the mode and affinity of binding between receptors and ligands, to predict the position and affinity of a ligand (small molecule) at the binding site of a receptor (macromolecule) (Ferreira et al. 2015). This technology has been widely used in drug design research in recent years. Furthermore, the introduction of reverse/backward molecular docking technology (Chen and Zhi 2001) has the potential to greatly improve drug target predictive power as well as an understanding of the related molecular mechanism for drug design. In silico methods are becoming useful and important in calculating the "n" number of structural and functional properties of various molecules. In silico methods are used to create and analyze clinical drugs (Taboureau and Steen Jorgensen 2011; Maryanty et al. 2020). The purpose of ligand–protein docking is to predict a ligand's most common binding mode(s) with a protein with a known three-dimensional (3D) structure (Deng et al. 2004). A ranking function must be used to rate the different candidate binding types, and a search method must be used to explore the state variables in all docking methods(Morris and Lim-Wilby 2008).

10.1.1.1 Key Concept of Molecular Docking

The very first docking methods are primarily based on Fischer's lock-and-key presumption, which explained that both the ligand and the receptor can be dealt with as rigid bodies and that their affinity is directly proportional to a geometric fit between their shapes (Tripathi and Bankaitis 2017). Later, Koshland's induced-fit

theory explained that the ligand and receptor should be dealt with as flexible during docking (Koshland Jr 1963; Hammes 2002).

10.1.1.2 Steps Involved in Molecular Docking

10.1.1.2.1 Protein Preparation

The 3D structure of the protein can be retrieved from the Protein Data Bank (PDB); the retrieved structure is then be pre-processed. According to the parameters available, this will allow for the removal of water molecules from the cavity, the stabilization of charges, the filling of lacking residues, side-chain formation, etc. (Chaudhary and Mishra 2016).

10.1.1.2.2 Protein Active Site Prediction

After protein preparation, the binding site of the protein can be predicted. The receptor may have several active sites, but only one in the query should be chosen. If present, water molecules and heteroatoms are mostly omitted (Ghersi and Sanchez 2009).

10.1.1.2.3 Ligand Preparation

Ligands can be recovered from databases such as ZINC and Pub Chem, or they can be sketched using the Chem sketch tool.2000. Recently, molecular docking as an approach has been gaining attention for predicting the preferred orientation of a ligand against a receptor (Protein) to form a stable complex (Lengauer and Rarey 1996). Using scoring functions, preferred orientation could be used to predict the strength of the connection or binding affinity between ligand and protein. Docking is frequently used to evaluate the predictive orientation of drug candidates against target proteins to predict the affinity and activity of the ligand. The method of studying the intermolecular interaction between two molecules in silico is known as molecular docking. The macromolecule serves as the protein receptor in this process. The ligand molecule, which can act as an inhibitor, is a macromolecule (Kitchen et al. 2004).

Thus, considering the above facts in the present study rice straw (RS), a LCB was used as a substrate for fungal growth. Keeping in mind the importance of xylanase in practical and industrial applications, the work has been formulated for determining xylanase activity in presence of xylan by two fungal strains *Rhizomucor variabilis* (Ac. No. KC602326.1) and *Penicillium* sp., (Ac. No. FJ430745.1). Then the xylanase (supernatant) from both the strains were analyzed for its potential to clarify fruit juice and deinking of newspaper via experimental and in silico analysis (Fig. 10.1).

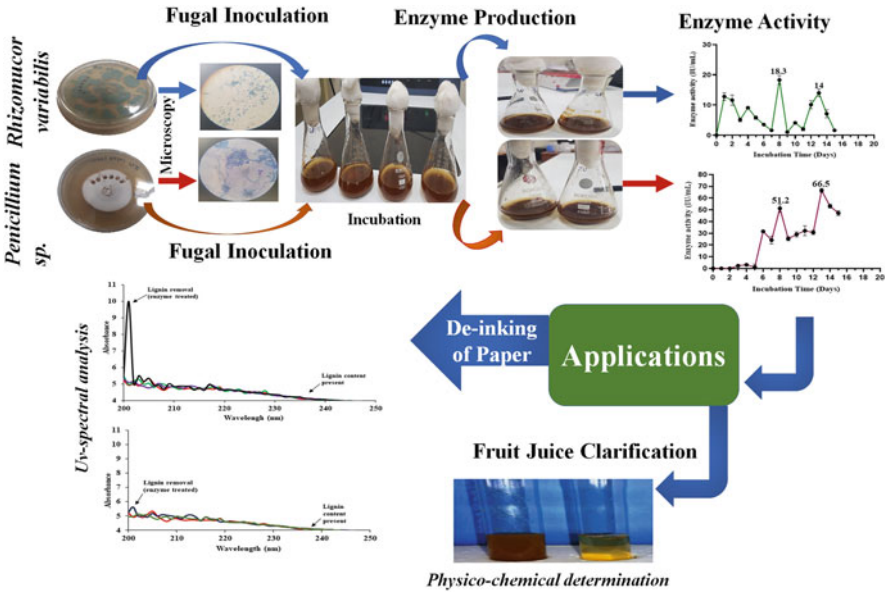


Fig. 10.1 Work plan for the present investigation

10.2 Material and Methods

10.2.1 Sample Strains, Substrates, and Chemicals

Xylanase enzyme was produced from in-house fungal culture previously identified as *Rhizomucor variabilis* (Ac. No. KC602326.1) and *Penicillium* sp., (Ac. No. FJ430745.1). Substrate (rice straw) was collected from fields of Rajasthan, and all the chemicals used in this study were of analytical grade and were purchased from Hi-media, CDH, Merck, and SRL.

10.2.2 Culture Plate Characterization and Microscopy

The provided in-house fungal cultures were characterized phenotypically and visualized morphologically using microscopy (Motic, China). Lactophenol Cotton Blue mounting (LPCB) was used to visualize fungal strains microscopically. After mounting the fungal strains, they were examined under a light microscope at 40X.

10.2.3 Xylanase Profiling Using Rice Straw Under Submerged Fermentation

Xylanase profiling was carried out using in-house fungal cultures *Rhizomucor variabilis* and *Penicillium* sp. The fermentation media used for production of xylanase was according to Bhardwaj et al. (2017), i.e., Mendel's Stenberg Basal Salt medium (MSBS) (g/L): 1% w/v RS (rice straw), 0.3 $MgSO_4$, 1.0 $CaCl_2$, 3.5 $(NH_4)_2SO_4$, 2.0 KH_2PO_4, 0.3 NH_2CONH_2, 5.0 $FeSO_4$, 1.6 $MnSO_4$, 1.4 $ZnSO_4.7H_2O$, 2.0 $CoCl_2$, and 1.0 peptone (Bhardwaj et al. 2017). The submerged fermentation was carried out in an Erlenmeyer flask (250 mL) containing media (50 mL) and inoculated with 3 cubes (6 mm) of 7 days old fungus culture and incubated at 28 ± 2 °C in static conditions. The experiments for both strains were performed in duplicates. The culture supernatant was withdrawn at a regular interval of 24 h for 15 days under aseptic conditions (Fig. 10.2). Followed by determining the xylanase activity and protein concentration determination using Lowry's method described as follows:

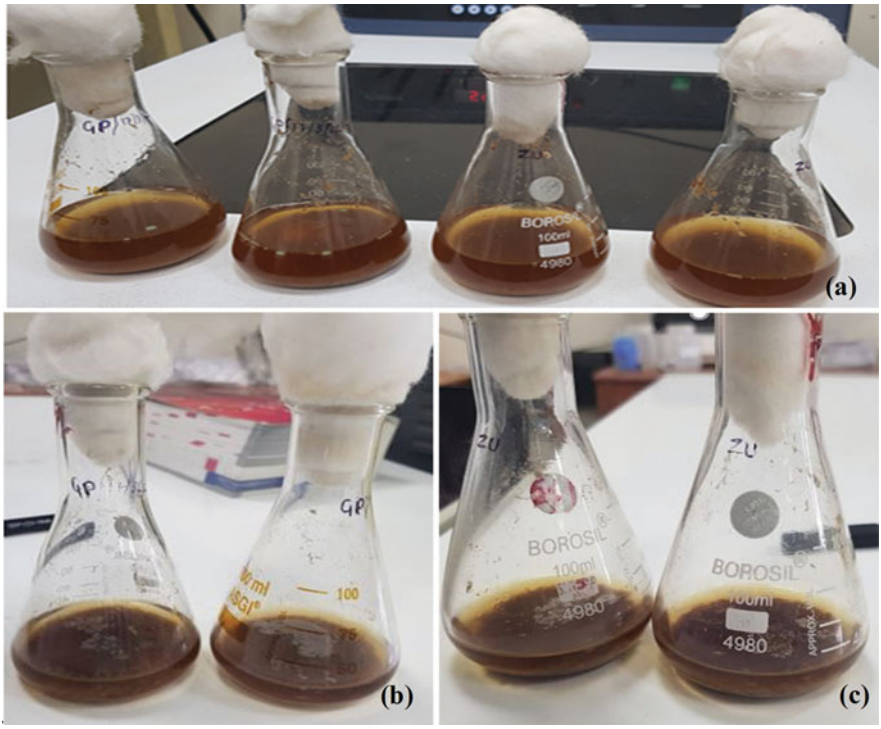


Fig. 10.2 (**a**) Fungal inoculation at first day, (**b**) flask having *Rhizomucor variabilis* growth at 15th day, and (**c**) flask having *Penicillium* growth at 15th day

10.2.3.1 Xylanase Assay

The xylanase activity was measured by following the method of Bailey et al. (1992). In this method 1% birchwood xylan solution was used as a substrate. The release of reducing sugars in 30 min at 50 °C, pH 4.8 (0.05 M citrate buffer) was measured as xylose equivalents and was estimated by the 3, 5-dinitrosalicylic acid (DNSA) (Miller 1959). The assay mixture consisted of 250 µL of sample and 250 µL of 1% w/v xylan solution (1 g/100 mL of 0.05 M Na-acetate buffer at pH 4.8) and was incubated in the water bath at 50 °C for 30 min. After 30 min, the reaction was terminated by adding 500 µL of DNSA and incubated for 10 min at 95 °C. And after 30 min, the absorbance was taken at 540 nm using a spectrophotometer and the amount of sugar released by the enzyme was determined.

10.2.3.2 Calculation of Xylanase Activity

One unit (U) of xylanase activity was defined as the amount of enzyme that produced 1 µmol min^{-1} of xylose (Corrêa Junior et al. 2022). Specific activities are expressed as units per milligram of protein. The enzyme activity of the sample can be analyzed by the optical density obtained from a UV-visible spectrophotometer. Enzyme activity using O.D. can be calculated using this formula:

$$\text{Enzyme activity}\left(\frac{U}{\text{mL}}\right) = \frac{\text{O.D.} \times \text{D.F}}{\text{Total volume} \times \text{Reaction time} \times \text{Slope(standard)}}$$

where O.D. is optical density, and D.F is dilution factor (Mandels and Weber 1969).

10.2.3.3 Protein Estimation by Lowry's Method

The "Lowry Assay" as per Lowry (1951) is the most commonly used method for estimation of the total amount of proteins (already in solution or easily soluble in dilute alkali) in biological samples. The proteins were first pre-treated in alkali solution with copper ion, and then the aromatic amino acids in the treated sample reduced the phosphor-molybdate-phospho-tungstic acid present in the Folin's reagent. This reaction's end product is blue. The absorbance at 750 nm after the end product of the Folin reaction against a standard curve of a selected standard protein solution can be used to estimate the amount of proteins in the sample (in our case; Bovine Serum Albumin-BSA- solution). To create the protein standard curve, different concentrations (mg/mL) of BSA stock (in the range 0.1–1.0) were added to 1.4 mL of Lowry's reagent and incubated for 20 min at room temperature. Then 0.2 mL of Folin's reagent was added and incubated for another 30 min in the dark. The standard protein curve was plotted using their absorbance. For protein estimation, 500 µL of sample and 700 µL of Lowry's reagent were mixed in a test tube and kept at room temperature for 20 min. After that, 100 µL of 1 N Folin's reagent was

added and kept for incubation in dark for 30 minutes before their respective absorbances were read at 750 nm.

10.2.4 Application of Xylanase in Fruit Juice Clarification and Deinking of Paper

10.2.4.1 Fruit Juice Clarification

Fruit juice clarification using xylanase was determined, and their physicochemical characteristics were taken into account by comparing the untreated tomato juice (control) with treated tomato juice (experimental).

10.2.4.1.1 Preparation of Tomatoes Pulp and Enzyme Treatment

Fresh tomatoes were purchased from the local market of Bandarsindri, Kishangarh, Ajmer (Rajasthan) and thoroughly washed with distilled water. Then puree was prepared by using a mixer, in order to achieve a smooth pulp. To separate the pulp from the juice, the pulp was filtered through muslin cloth and centrifuged at 5000 rpm for 10 min in the centrifuge (REMI TM 80 PLUS). A 1.5 mL supernatant of juice was taken in an Eppendorf tube and treated with 200 μL of xylanase by both the in-house fungal cultures having the highest enzyme activity of the days listed in Table 10.1.

Table 10.1 The enzyme production by *Rhizomucor variabilis* using xylan as a substrate on each day of production

Incubation (days)	Enzyme activity (IU/mL)	
	Rhizomucor variabilis	*Penicillium sp.*
1	12.8	N.D
2	11.6	N.D
3	5.1	2.3
4	9.1	3.1
5	5.8	28.8
6	3.5	31.6
7	1.6	24.1
8	18.3	51.2
9	1.0	25.3
10	4.1	29.0
11	2.0	32.1
12	10.1	30.7
13	14.0	66.5
14	7.1	53.1
15	1.6	47.2

10.2.4.1.2 Physio-Chemical Characteristics Determination of Tomato Juice

The tomato juice of both xylanase-treated and untreated tomatoes (control) was tested for sugar reduction, clarity, and filterability. After centrifugation and filtration, the total yield was expressed as a percentage (%) (volume of juice per 100 g of pulp). The clarity percentage was calculated by comparing transmittance to water using a UV-visible spectrophotometer. The DNS method was used to compare sugar reduction to the xylose standard curve. Filtering juice through filter paper determined the filterability per minute (Ullah et al. 2019).

10.2.4.1.3 Reducing Sugars Determination

Reducing sugars released after the enzymatic treatment of the pulp were determined through a modified Bailey's method using DNS acid as a reaction terminating reagent. Reducing sugar was checked by the DNS method against the xylose standard curve (Ullah et al. 2019).

10.2.4.1.4 Clarity Determination

The clarity of the juice was determined by measuring the percent transmittance (T%) at a wavelength of 660 nm spectrophotometer taking distilled water and untreated tomato juice as a reference. The percent transmittance was considered a measure of juice clarity (Nagar et al. 2012).

$$\text{Clarity}(\%) = (X_t - X_c/X_c) \times 100$$

where X_t = transmittance of test and X_c = transmittance of control (Rosmine et al. 2017).

10.2.4.1.5 Filterability

Filterability (/s) was calculated by reversing the time required to filter enzyme-treated juice using Whatman No. 1 filter paper (Ullah et al. 2019).

10.2.4.2 Deinking of Newspaper

Newspaper pulp for evaluating the deinking ability of xylanases was prepared as described by Virk et al. (2013) with some modifications. Newspapers were crushed and soaked in water at 60 °C in a water bath containing Tween 80 (0.1% w/v) for 2 h before being disintegrated in a domestic mixer. The extra water in the pulp was strained out, and the pulp was oven-dried at 50 °C. The dry newspaper pulp (6.0 g)

was soaked for 30 min in 60.0 mL of distilled water (pH 5.5) to obtain a pulp with consistency. After that 1 mL, the effluent of newspaper pulp is taken into Eppendorf tube and was treated with 200 µL supernatant of *Rhizomucor variabilis* and *Penicillium sp.* having enzyme activity of first 3 days that is Day 1, Day 2, Day 3 in (Table 10.1) and incubated at 55 °C for 20 min on an orbital shaker (150 rpm) for deinking reaction (Desai and Iyer 2016). After the preparation of newspaper pulp, the UV spectrum analysis was performed after 24 h xylanase treatment at room temperature with UV-Vis Spectrophotometer (Shimadzu-2600, Japan). The effluent was examined under the visible range (200–700 nm) to determine the release of lignin-related compounds during deinking.

10.2.5 In silico Enzyme Docking

In the present study, the software used was Mcule's 1-click docking web-based analysis platform (https://mcule.com/apps/1-click-docking/). The enzymes' PDB files were downloaded and docked with standard ligands for all the polymers under consideration. The enzyme xylanase of *Penicillium chrysogenum* and *Talaromyces funiculosus* was considered as model xylanase obtained from *Penicillium* sp. and *Rhizomucor variabilis,* respectively. The xylanase enzymes were docked with xylan, xylose, xylobiose, xylotriose, cellobiose, and lignin. The docking score pattern, which reflects the relatively linear relationship of the binding energy with the docking score, was used to analyze the affinity of ligands toward the enzyme. The Vina filter is used in 1-Click docking to dock multiple ligands into a single target. 3-D structures of the tested substrates, cellulose, hemicellulose, and lignin were taken from PubChem by using their InChIKey ID. Furthermore, Pymol (https://pymol.org) was used for better visualization or orientation, where a downloaded PDB file from Mcule is uploaded and visualized. Different colors were used for a clear representation of ligand and receptor, which aids in easy differentiation of ligand and receptor.

10.2.5.1 Molecular Docking of Xylanase from *Penicillium chrysogenum* as Model Xylanase Obtained from *Penicillium* sp.

The PDB file of coordinates for *Penicillium chrysogenum* xylanase were downloaded from Uniprot (https://www.uniprot.org), and the InChIKey ID for the substrate was obtained from Pubchem (https://pubchem.ncbi.nlm.nih.gov) and uploaded to Mcule for docking to see the docking score or binding energy of enzyme–ligand interaction and for better visualization, Pymol was used.

10.2.6 *Molecular Docking of Xylanase from* Talaromyces funiculosus *as Model Xylanase Obtained from* Rhizomucor variabilis

Since *Rhizomucor variabilis* is a newly sequenced organism with limited available information or data in the literature, the coordinates are not available at Swissprot or Uniprot. The most used and most efficient method for characterizing newly determined sequences is sequence similarity searching, typically with BLAST. By detecting excess similarity—statistically significant similarity that reflects common ancestry—sequence similarity searches can identify "homologous" proteins or genes. Searching for homologous sequences using sequence similarity is one of the first and most informative steps in any analysis of newly determined sequences (Pearson 2013). So, to identify similar sequenced organisms with an available Accession number that is KC602326.1, the nucleotide sequence in FASTA format is obtained from NCBI (http://www.ncbi.nlm.nih.gov) and submitted to blastx which searches protein databases using a translated nucleotide query. This helps in identifying similarly sequenced organisms by considering query length and providing the percent identity of the homologous organism for the *Rhizomucor variabilis*. After obtaining a list of the most similar ten sequenced organisms with their hypothetical proteins, *Penicillium italicum* was selected for in silico study and searched on Uniprot for *Penicillium italicum* where unreviewed xylanase from *Penicillium italicum* is present and its base model is constructed with the help of reviewed xylanase of *Talaromyces funiculosus*, as previously reported by bioinformaticians. Following that, in the 3-D structure database, there is SMR (Swiss Model Repository) with the ID Q9HFH0, which is used to construct a model with coordinates and this gives an idea about binding sites. Later downloaded coordinates in PDB format are used for molecular docking in Mcule with their respective substrates and give the binding score for enzyme–ligand interaction, the enzyme–ligand interactions were visualized using Pymol.

10.3 Results and Discussions

10.3.1 *Culture Plate Characteristics and Microscopic Visualization*

The fungal strain *Rhizomucor variabilis* on Potato Dextrose Agar (PDA) medium plates had powdery greenish color colonies (Fig. 10.3a). LPCB mounting was used to identify the fungal isolates under the microscope, and they appeared as spores (Fig.10.3b) under the microscopic and belongs to zygomycetes as reported earlier by (Richardson 2009).

The strain *Penicillium* sp., appearing on PDA plates were velvety and smooth colonies with pure white color. The colony's reverse side was also white (Fig.10.3c) on the PDA plate. LPCB mounting was used to identify the fungal isolates under the

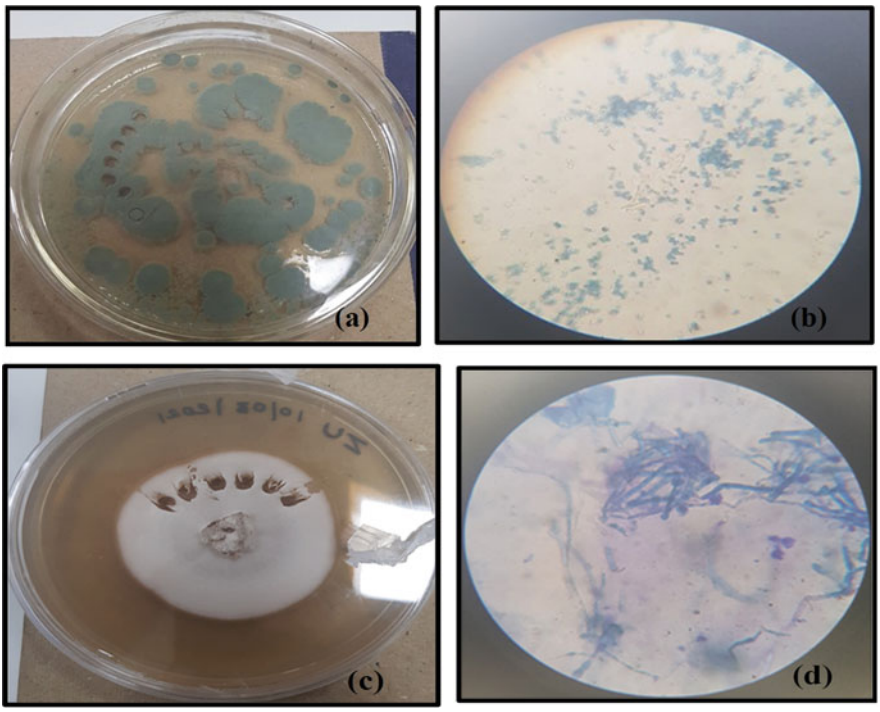


Fig. 10.3 (**a**) Colony morphology of *Rhizomucor variabilis*, (**b**) microscopic visualization of *Rhizomucor variabilis*, (**c**) colony morphology of *Penicillium sp*., (**d**) microscopic visualization of *Penicillium* sp.

microscope. *Penicillium sp.* appeared as branched mycelia with septate hyphae (Fig 10.3d). Conidiophores germinated on the mycelium, and conidiospores were observed and the microscopic observations were similar to earlier reported morphological features of *Penicillium* genera belonging to the ascomycetes phylum (Visagie et al. 2005).

10.3.2 Xylanase Assay

The samples from the production media were quantified for xylanase activity using the xylose standard curve. The enzyme activity was assayed by Miller's method.

10.3.2.1 Xylanase Activity of *Rhizomucor variabilis*

Rhizomucor variabilis xylanase activity clearly shows two peaks of enzyme activity with different incubation times. The first peak was observed on the eighth day of

incubation (early stationary phase), and the second peak was observed on the 13th day of incubation (late stationary phase). On the 8th and 13th day, enzyme activity was 18.300 and 13.967 IU/mL, respectively (Table 10.1 and Fig. 10.4). These findings were like those obtained for xylanase from other microbial sources (Kurrataa'Yun et al. 2015).

10.3.2.2 Xylanase Activity of *Penicillium* sp.

Two peaks of xylanase activity of *Penicillium sp.,* on the 8th and 13th day are observed, i.e., 51.042 and 66.319 IU/mL, respectively. This is similar to *Rhizomucor variabilis*, where it showed two high peaks of xylanase activity on the 8th and 13th day as well (Table 10.1).

Xylanase activity of both the in-house fungal cultures shows almost the same trend of having 2 peaks that are a characteristic feature of fungal enzyme production reported in the literature. The first peak corresponds to the early stationary phase, and the second peak corresponds to the late stationary phase. The findings were similar to the early reported data for *Trichoderma* strains (Silva et al. 2015).

Multiple enzyme forms are a common phenomenon in nature and can be regarded as a specialized function of microbes to accomplish extra efficient hydrolysis of heterogeneous substrates (Wong et al. 1988). Multiple peaks could also be caused by isoenzyme and the presence of polysaccharides in the medium (Sener 2015; Chalisya et al. 2020). Also, Jermyn (1962) discovered that combining aryl-β-glucosidase and polysaccharide resulted in multiple peaks at enzyme activity measurements This has been commonly reported in fungal enzyme production

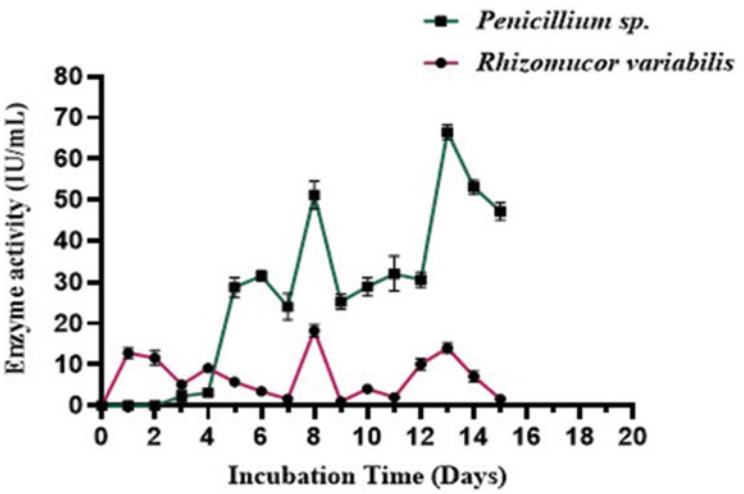


Fig. 10.4 Comparative analysis of enzyme production of both in-house fungal cultures using rice straw as substrates

systems (Kurrataa'Yun et al. 2015). The other probable reason for the two peaks may be that the fungus is utilizing primary metabolites, and utilization of primary metabolites results in the formation of a second peak. That is why there are two peaks of enzyme activity.

The graphs of xylanase activity for both the in-house fungal cultures show two distinct peaks on 8th and 13th day but much higher xylanase activity was obtained for *Penicillium sp.,* as compared to *Rhizomucor variabilis*.

10.3.3 Protein Concentration by Lowry's Method

The samples withdrawn on each day were quantified using Lowry's method.

10.3.3.1 Protein Concentration of *Rhizomucor variabilis*

Total cellular protein (an indicator of cell growth) reached a peak on the 11th day of cultivation. Protein concentration of *Rhizomucor variabilis* was 0.27 mg/mL on the first day and shows a very less variation in concentration till the fourth day. After the fourth day, there was a sudden increase in concentration which was 1.19 mg/mL (Fig. 10.5) and shows the highest concentration on the 11th day which was 1.23 mg/mL of protein concentration.

10.3.3.2 Protein Concentration of *Penicillium sp.*

The protein concentration of *Penicillium sp.* was 0.23 mg/mL on the first day and remains constant till the fourth day. After, the fourth day, it showed a spike in

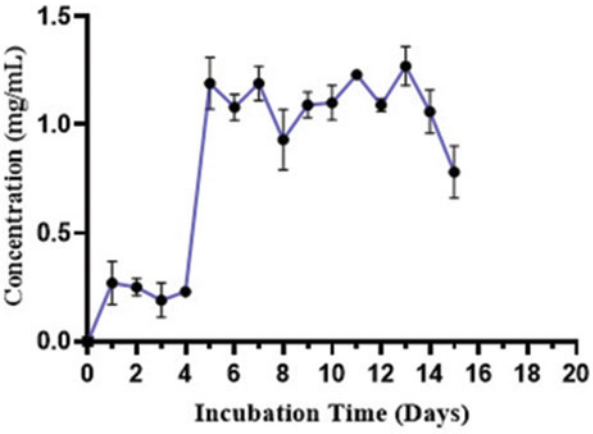


Fig. 10.5 Protein concentration over the production period of *Rhizomucor variabilis*

concentration, i.e., 0.99 mg/mL on the fifth day and showed highest protein concentration on the 12th day which was 1.34 mg/mL (Fig. 10.6).

10.3.4 Findings of Fruit Juice Clarification

10.3.4.1 Reducing Sugar Content

The xylanase enzyme was successfully used against tomato juice, that improved its properties. Enzymatic treatment improved the release of reducing sugars when it was presented in a 1:1 of enzyme extract to juice and begins to decrease when the amount of juice is gradually increased while the amount of enzyme remains constant. As a result of xylan hydrolysis, enzymatic treatment increases the release of reducing sugars such as xylose and other xylooligosaccharides (Fig. 10.7) (Bajaj and Manhas 2012; Sharma et al. 2021)

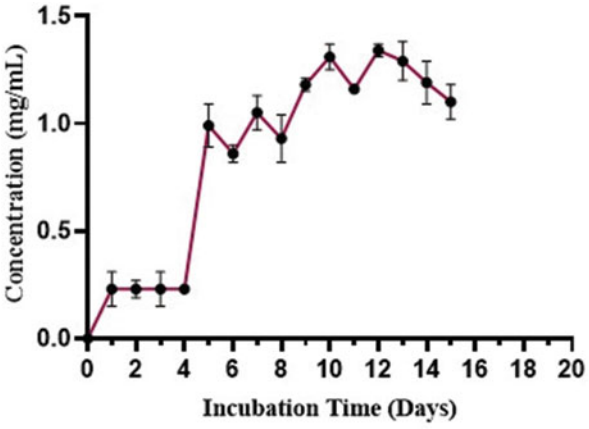


Fig. 10.6 Protein concentration over the production period of *Penicillium sp.*

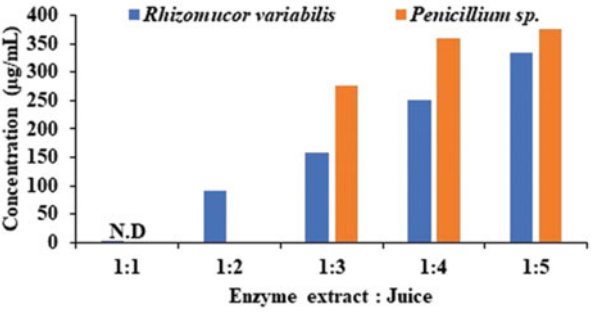


Fig. 10.7 Comparative analysis of reducing sugar content of tomato juice clarification after xylanase treatment for 24 h. *N.D* Not Detected

10.3.4.2 Clarity

Clarity is considered the direct measure of transmittance, and the data obtained by experimental is summarized in Table 10.2. As the amount of juice increased and the amount of enzyme was kept constant, subsequently the clarity reduced from 1:1 to 1:5 of enzyme extract to juice ratios. Similar findings were observed by Sharma and Chand (2012) in Mausambi and orange fruit juice clarification.

10.3.4.3 Filterability

The filterability of 2 mL of untreated tomato pulp (control) is 1 min 12 s and 2 ms, while enzyme-treated pulp has a filterability of 53 s. A similar type of observation was found by Gaur et al. (2015) that an increase in filterability of tomato juice could be possible as a result of enzymatic hydrolysis of xylan.

10.3.5 Findings of Deinking

The effluent obtained after enzymatic deinking of newspaper exhibited peaks corresponding to the UV spectra (200–700 nm). In the case of *Rhizomucor variabilis,* peaks between 200 and 205 nm, indicating the discharge of lignin-related contents in the deinked effluent (Fig. 10.8a). On the other side, *Penicillium sp.* does not exhibit a sharp peak (Fig. 10.8b), which shows a less/no effective enzyme deinking property.

Singh et al. (2012) reported deinking of school wastepaper and newspaper waste, where the effluent collected after newspaper waste deinking was characterized. Xu and colleagues in 2009 reported two peaks of lignin-related contents at 205 and 280 nm (Xu et al. 2009), thus suggests the loss of lignin-related components either due to the presence of ligninolytic enzymes in the crude extract of fungal strains (*Rhizomucor variabilis*) or by the breakdown of hemicellulosic (xylan constituent) component making lignin freely accessible.

Table 10.2 Effect of xylanase treatment on clarity (% transmittance) of tomato juice clarification

	% Transmittance	
Enzyme extract: juice	*Rhizomucor variabilis*	*Penicillium sp.*
Control	100	100
1:1	57.1	78.6
1:2	53.4	68.4
1:3	44.9	58.2
1:4	41.2	50.8
1:5	38.7	45.8

Control Distilled water and untreated juice having no enzyme

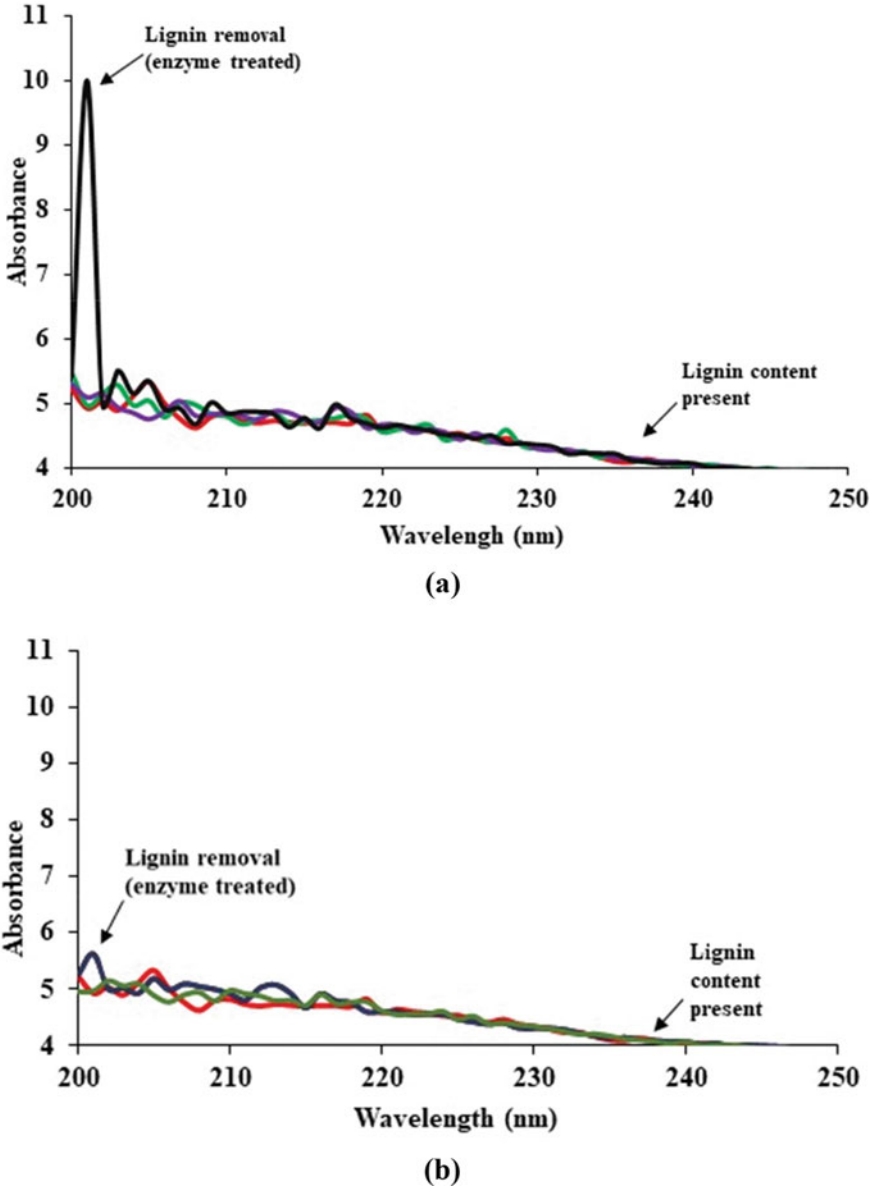


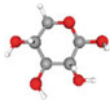
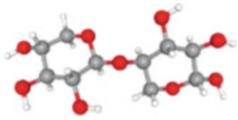
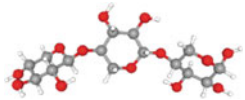
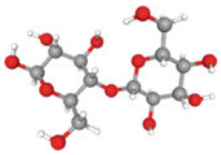
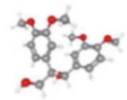
Fig. 10.8 UV-visible spectra of effluent obtained after xylanase treatment of (**a**) *Rhizomucor variabilis*, (**b**) *Penicillium sp.*

10.3.6 In Silico Enzyme Docking

Xylanases have gained popularity in the production of bioenergy from LCB as well as other industrial applications. Xylanase is essential for the hydrolysis of hemicellulose (present in plant cell wall components) into XOS and xylose. The production of xylanase by fungi has a significant advantage over other microorganisms in terms of preserving energy for growth. The current in silico work focuses on the molecular characterization of xylanase and its binding energy with the different substrates. Recognition of catalytically essential active site residues is necessary to understand the mechanism of enzyme and substrate binding, classification of enzymes, and forthcoming targeted bioengineering of any protein (Dimitriou et al. 2017).

For the structure of the ligand refer to Table 10.3. Visualized pose of the ligand with the enzyme was also obtained during the procedure in Figs. 10.9 and 10.10.

Table 10.3 Structure of ligands

S. No.	Name of ligand	3-D structure of ligand
1.	Xylan	
2.	Xylose	
3.	Xylobiose	
4.	Xylotriose	
5.	Cellobiose	
6.	Lignin	

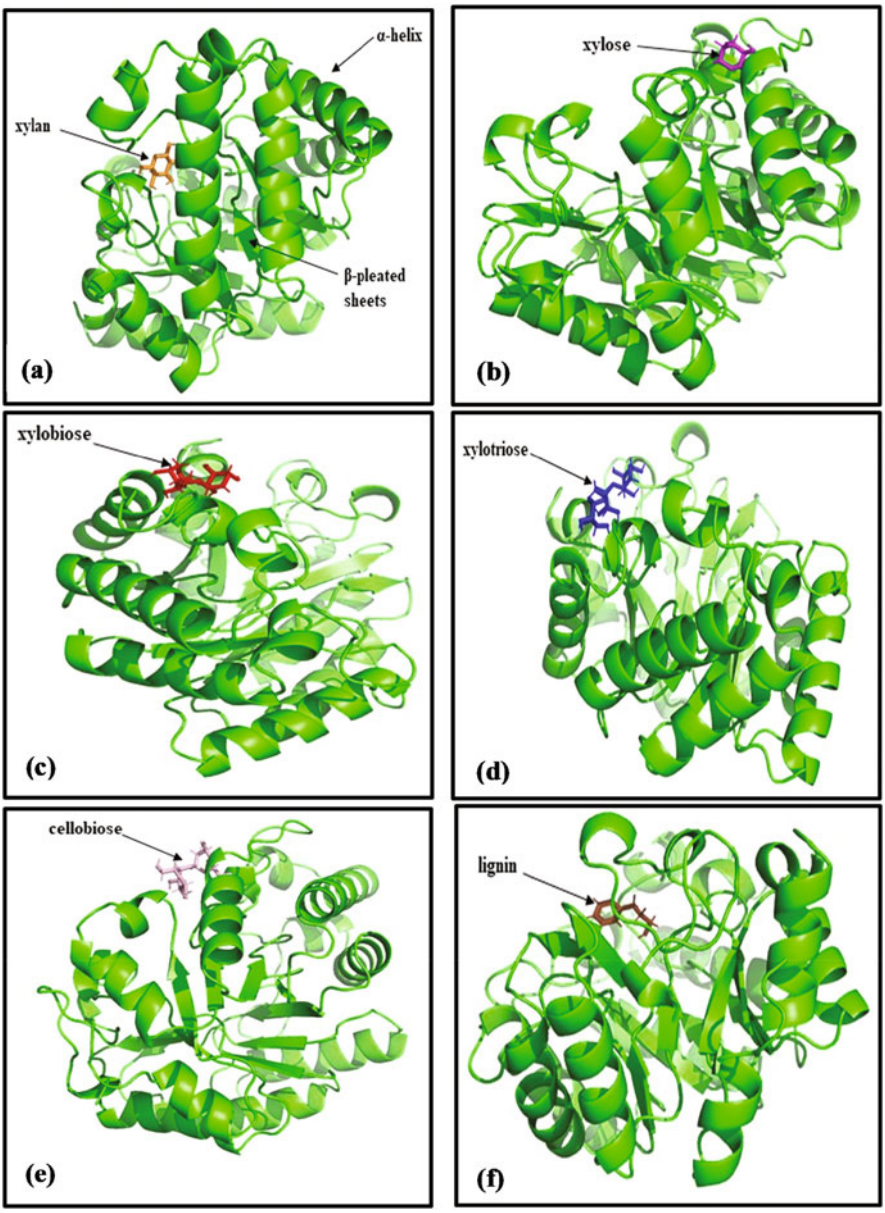


Fig. 10.9 Docking orientations of *Penicillium chrysogenum* xylanase Q6PRW6 with (**a**) xylan, (**b**) xylose, (**c**) xylobiose, (**d**) xylotriose, (**e**) cellobiose, and (**f**) lignin

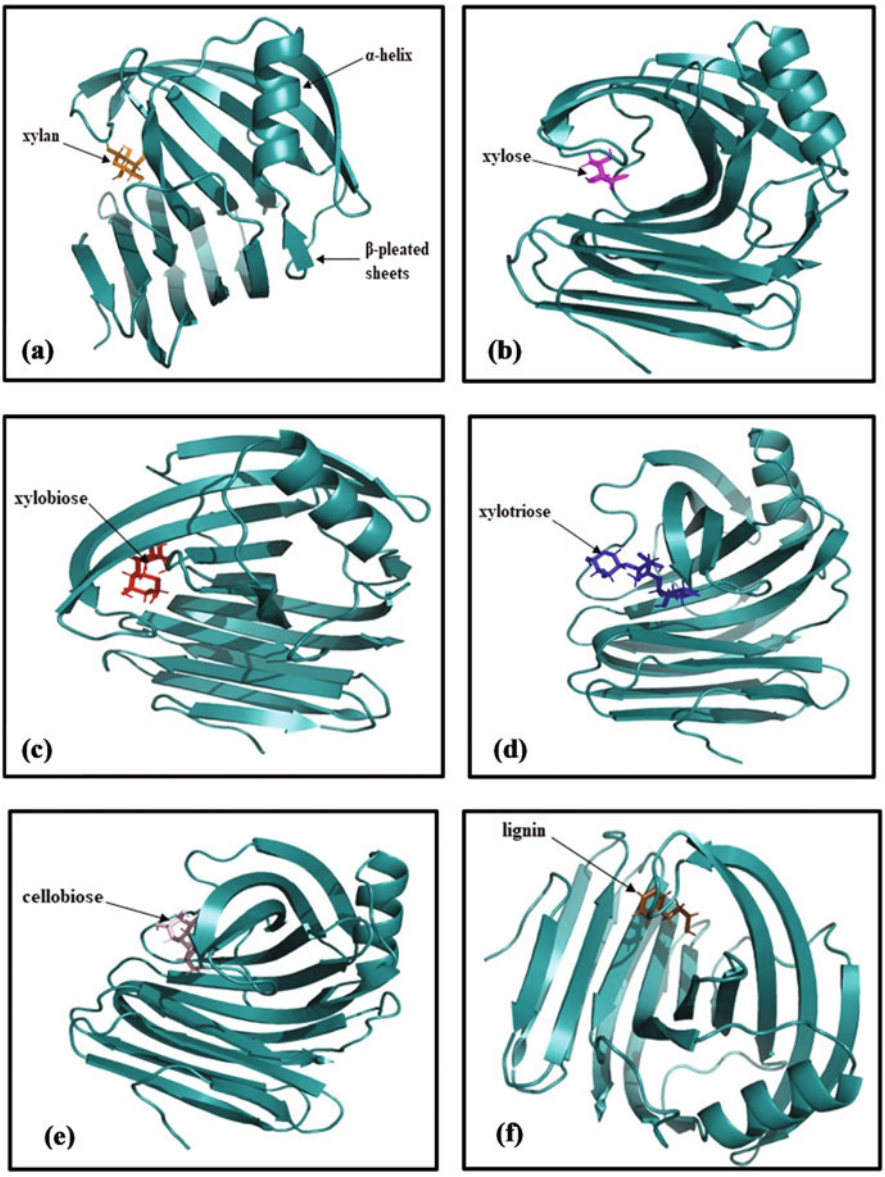


Fig. 10.10 Docking orientations of *Talaromyces funiculosus* xylanase Q9HFH0 with (**a**) xylan, (**b**) xylose, (**c**) xylobiose, (**d**) xylotriose, (**e**) cellobiose, and (**f**) lignin

10.3.6.1 Molecular Docking of *Penicillium* sp. by Considering a Base Model of *Penicillium chrysogenum* Xylanase

Penicillium chrysogenum xylanase when docked against ligands generates a docking score as listed in Table 10.4. *Penicillium chrysogenum* xylanase when docked with xylan, xylose, xylobiose, xylotriose, cellobiose, and lignin generated docking scores. The docking score aligns with the binding and suggests that the binding of the enzyme xylanase has the highest energy in the case of xylotriose and the lowest in the case of xylose. This observation suggests that the xylanases have the best affinity with xylanase and the lower range with lignin (Table 10.4). Dodd and Cann (2009) and Bhardwaj et al. (2019) confirmed our findings stating that xylanase aids in the degeneration of linear polysaccharides such as xylan and converts them to xylose.

10.3.6.2 Molecular Docking of *Rhizomucor variabilis* by Considering a Base Model of *Talaromyces funiculosus*

Talaromyces funiculosus xylanase generated a docking score of −5.4 with xylan, −4.9 with xylose, −5.8 with xylobiose, −7.0 with xylotriose, −6.4 with cellobiose, and −5.6 with lignin. Binding site coordinates are x = −6.614; y = −4.375; and z = 17.749 which were obtained by blind docking (Table 10.5). When the protein and ligand comes together, the negative score mimics the potential energy change. This means that a very negative score indicates a strong binding, whereas a less negative or even positive score indicates a week or non-existent binding.

Mcule was used to dock selected xylanase enzymes and ligand datasets for each approach. Our results show that the Mcule docking software can select well-known substrates like xylan, xylose, xylobiose, xylotriose, cellobiose, lignin, and others (Tables 10.4 and 10.5). Further support for the in silico study comes from the

Table 10.4 Enzyme–ligand docking of *Penicillium chrysogenum* xylanase with different substrates

S. No.	Substrates	InChIKey ID of ligand	Target protein	Docking score
1.	Xylan	HEHIOFQJTRFOKM-ASQQECOQSA-N	Q6PRW6 (xylanase)	−5.0
2.	Xylose	SRBFZHDQGSBBOR-IOVATXLUSA-N	Q6PRW6 (xylanase)	−4.4
3.	Xylobiose	LGQKSQQRKHFMLI-WSNPFVOISA-N	Q6PRW6 (xylanase)	−4.8
4.	Xylotriose	JCSJTDYCNQHPRJ-FDVJSPBESA-N	Q6PRW6 (xylanase)	−5.8
5.	Cellobiose	GUBGYTABKSRVRQ-QRZGKKJRSA-N	Q6PRW6 (xylanase)	−5.2
6.	Lignin	RWYKESRENLAKMN-UHFFFAOYSA-N	Q6PRW6 (xylanase)	−5.4

Table 10.5 Enzyme–ligand docking of *Talaromyces funiculosus* xylanase with different substrates

S. No.	Substrates	InChIKey ID of ligand	Target protein	Docking score
1.	Xylan	HEHIOFQJTRFOKM-ASQQECOQSA-N	Q9HFH0 (xylanase)	−5.4
2.	Xylose	SRBFZHDQGSBBOR-IOVATXLUSA-N	Q9HFH0 (xylanase)	−4.9
3.	Xylobiose	LGQKSQQRKHFMLI-WSNPFVOISA-N	Q9HFH0 (xylanase)	−5.8
4.	Xylotriose	JCSJTDYCNQHPRJ-FDVJSPBESA-N	Q9HFH0 (xylanase)	−7.0
5.	Cellobiose	GUBGYTABKSRVRQ-QRZGKKJRSA-N	Q9HFH0 (xylanase)	−6.4
6.	Lignin	RWYKESRENLAKMN-UHFFFAOYSA-N	Q9HFH0 (xylanase)	−5.6

experimental data showing that xylanases can reduce the predicted potential targets and result in the formation of usable products and have great potential in various applications. As a result, it confirms Mcule's ability to identify well-known enzyme binders from a pool of ligands

10.4 Conclusion

In the modern era, concern about the preservation of our resources and the environment has sparked a surge in investment in the production of microbial enzymes. Because of their use in a large number of applications, industrial enzymes, especially those of microbial origin, are in high demand. Due to the technological advancements in chemical technologies, where mass production of a wide range of products has ultimately resulted in resource depletion and severe hazardous waste disposal issues, the need and demand for safer and more eco-friendly technologies have become essential. Reaction mediated by enzymes is appealing alternative to time-consuming and costly chemical methods. LCB has the potency to be used as a source of enzyme production. The use of LCB will solve the waste management problem because it is abundant and can be economically viable. In the present study, *Rhizomucor variabilis* (KC602326.1) and *Penicillium sp* (FJ430745.1) were used to produce xylanase from rice straw, a low-cost substrate. Furthermore, crude xylanases showed efficiency in fruit juice clarification and deinking of paper.

10.5 Significance and Future Aspects

India is the leading provider of agricultural and forestry-based sustainable resources, which are usually regarded as waste. Typically, these wastes, which are high in fermentable carbs, occupy land space and lead to ecological imbalances. On the other hand, India's industrial sector is diversifying, and it has huge potential to utilize enzyme-catalyzed methods to replace chemical methods. To justify both the industrial and environmental needs, profitable enzyme production from low renewable biomass is now a national preference. Presently, there are only two or three xylanase manufacturing companies in India; otherwise, we import xylanase preparations from other developed countries. Gaining a better understanding of xylanase in both practical and industrial sectors, the present work has been structured for the "production of xylanases using cost-effective substrate and its application in various fields." In addition, in silico work is done to find the best binding score that corresponds to the best fit substrate.

The market opportunity for xylanases is growing rapidly. The applicability of xylanases by substituting chemical-based methods is only feasible if adequate supplies are available at a low cost. High expectations are positioned in finding novel xylanase-producing microorganisms that have the adaptability to grow naturally and use cheap substrates for growth, as well as on new technologies. Until now, the potency of extremophilic xylanases from novel habitats has received little attention, which could open up new opportunities for xylanase-based industries. Developing a low-cost method for xylanase production is advantageous because, as previously stated, xylanase has a wide range of potential applications. Studies showed that hemicellulose conversion to value-added useful products via enzymatic and/or fermentation routes holds great promise for converting discarded and underutilized LCB to usable products. Thus this needs to be further exploited for future industrial applications.

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

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Chapter 11
Life Cycle Assessment of Thermochemical Conversion of Agro Residues

Abhijeet Anand and Priyanka Kaushal

Abstract Agro residues could be utilized as a fuel in solid form or by converting to liquid or gaseous fuel such as bio-oil and biogas. Fuel conversion of agro residues either involves biochemical conversion or thermochemical conversion processes. The biochemical conversion process includes anaerobic digestion and fermentation, whereas thermochemical conversion includes direct combustion, gasification, and pyrolysis. These technologies have been believed to produce drop-in grade fuels which show promising potential for decarbonizing the transportation sector and supplying clean energy as an alternative to fossil fuels. Recent advancements and large-scale demonstrations of these technologies show its readiness to replace/substitute the centuries-old fossil fuel-based energy supply system. However, it is essential to investigate the economic, technological, and environmental trade-offs of these technologies. Life cycle assessment is a tool to access and quantify the possible environmental impacts and sustainability of these technologies. In this chapter, the authors elaborate on biochar, syngas, and bio-oil conversion of agro residues and discuss its sustainability and possible environmental impacts.

Keywords Life cycle assessment · Agro residues · Pyrolysis · Biochar · Bio-oil

Abbreviations

CAGR	Compound annual growth rate
GDP	Gross Domestic Product
GHG	Greenhouse gas
GWP	Global warming potential
LCA	Life cycle assessment
MNRE	Ministry of New and Renewable Energy

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11.1 Introduction

Although India remains an agro-centric economy after independence in 1947, the country has achieved high industrial growth and development coupled with scientific achievements. Industrial and economic activities in India have expanded rapidly since the beginning of the globalization of the Indian economy in the early 1990s. Consequently, energy supply and electricity consumption in India have increased 200.73% to 919.44 Mtoe and 458.80% to 1309.44 TWh, respectively, in 2018 from 1990 (IEA 2020). Fossil fuels (coal, petrol, and diesel) have been the primary energy source for transportation and electricity generation and have a 70.61% share in the Indian energy basket. However, India does not have sufficient petroleum reserves, and domestic crude oil production has stagnant at about 32.5 MT since 2000–2001 (Central Statistics Office 2019).

On the other hand, coal production in India has increased to 730.87 MT in 2019–2020 but remains insufficient to meet the coal demand. India imported 25.38% coal and 87.59% crude oil to fulfill energy demand in 2019–2020. Coal and crude oil imports have increased at 13.17% and 5.76% CAGR, respectively, since 2000–2001. Increasing coal and crude oil imports impose a massive burden to exchequer account and poses a daunting threat to the country's energy security in the present geopolitical scenario. Coal and crude oil import value amounted to 123.08 billion USD in 2019–2020, which was 4.54% of the country's GDP. It is worth mentioning that there has been significant variation in crude oil import values since 2008–2009, which could be attributed to the high price volatility of crude oil in the international market due to the geopolitical situation. Trends in coal and crude oil imports have been shown in Fig. 11.1.

Fossil fuel-based transportation and electricity generation system have also been considered the most polluting globally. With a 3346.63 MT CO_2 eq. (6.84% of world total) Greenhouse Gas (GHG) emissions, India is the third-largest emitter after the USA and China (Ge et al. 2020). According to the WRICAIT report, the energy sector (72.45%) is the highest GHG emitter, followed by agriculture (21.47%) and industrial processes (4.44%) in 2018 in India. On the other hand, India has pledged to reduce its carbon emissions by 33–35% at the 2005 level and increase non-fossil-based energy resources to 40% of installed electricity generation facilities by 2030 (PIB 2016). Therefore, the country has started exploring its domestic renewable energy sources to reduce energy imports and fulfill its voluntarily yet legally binding commitment under the Paris climate deal in 2015.

Biomass such as agro residues, forest residues, and municipal solid wastes have been seen as promising renewable energy resources as an alternative to fossil fuels. Waste biomass can be converted to solid, liquid, and gaseous biofuels to replace the present fossil fuel-based energy system globally and in India. There have been various waste-to-wealth technologies, including thermochemical processes (combustion, gasification, and pyrolysis) and biochemical processes (fermentation, anaerobic digestion, and hydrolysis) for biomass conversion into a useful form of clean and green energy (Jiang et al. 2019; Goswami et al. 2020, 2021a). These technologies have continuously evolved, and few have been demonstrated at a large scale.

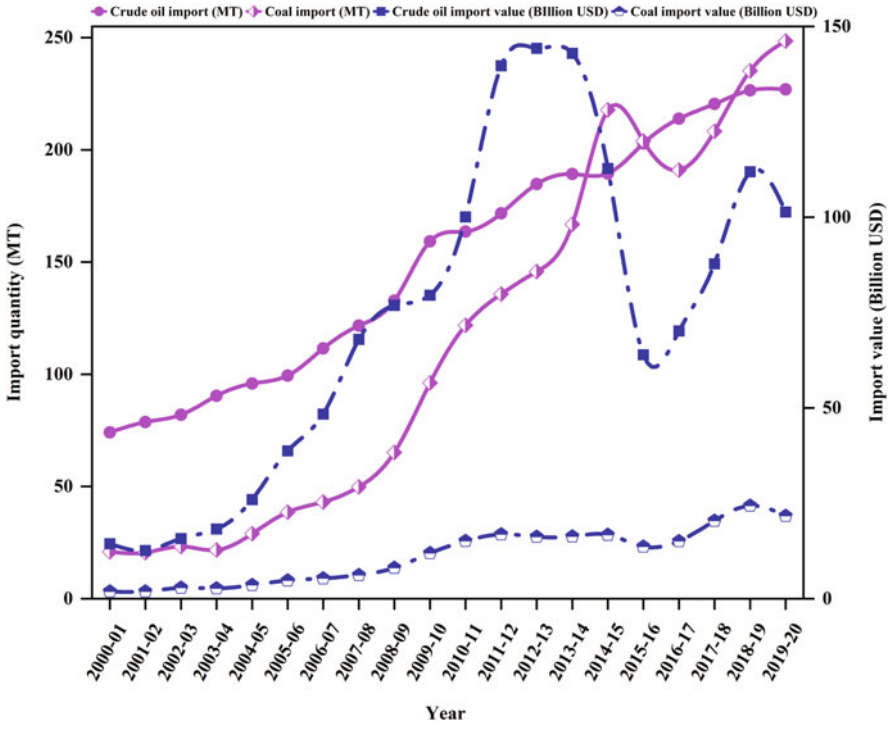

Fig. 11.1 Trends in crude oil and coal import quantity and its economic value during 1998–1999 to 2019–2020 in India (Data source: National Statistical Office 2021)

However, enormous efforts would be required to develop biomass–biofuel systems for techno-economically efficient production, conversion, and utilization of biofuels under environmentally sustainable conditions. Biorefinery models have been envisaged for biofuels production to make a sustainable society (nee'Nigam and Pandey 2009; Kumar and Verma 2020; Goswami et al. 2022). This chapter presents a comprehensive Life Cycle Assessment (LCA) of promising thermochemical technologies for agro residues valorization in India as a renewable energy source.

11.2 Agro Residues Availability

India is a country where approximately 65% of the population reside in rural area. Agriculture has been the front-runner in the Indian economy, contributing 17–18% of GDP. About 42.6% of the population is directly or indirectly involved in agriculture and allied activities (Directorate of Economics and Statistics 2021). Out of 328.7 Mha land area in the country, around 52.71% area is engaged in agriculture. Currently, India is the second-largest agro producer globally, and approximately 700 MT of food grains and other agro produce were harvested from

Table 11.1 Sown area, production, and yields of the principal crops in India (2019–2020) (Data source: Directorate of Economics and Statistics 2021)

Crop category	Crops	Area (Mha)	Production (MT)	Yield (kg/ha)
Cereal	Rice	43.78	118.43	2705
	Wheat	31.45	107.59	3421
	Maize	9.72	28.64	2945
	Bajra	7.52	10.28	1368
	Jowar	4.71	4.73	1005
	Other nutri cereals	2.07	3.83	1850
Oilseeds	Rapeseed and mustard	6.78	9.12	1345
	Soybean	12.09	11.22	928
	Sunflower	0.24	0.22	891
	Groundnut	4.89	10.1	2065
	Other oilseeds	3.04	2.76	908
Pulses	Lentil (Masur)	1.32	1.18	894
	Gram	10.17	11.35	1116
	Tur (Arhar)	4.54	3.83	842
	Other pulses	12.31	6.79	552
Cash crops	Sugarcane	4.57	355.70	77,893
	Cotton	13.37	6.033	451
	Jute and Mesta	0.68	1.784	2641

180 Mha sown area during 2019–2020. Primary agro produce could be categorized into four broad categories as follows:

(a) Cereals: Rice, Wheat, Maize, Bajra, Barley, Jowar, Small millet, and Ragi
(b) Oilseeds: Rapeseed and Mustard, Sesame, Linseed, Niger, Safflower, Soybean, Groundnut, and Sunflower
(c) Pulses: Tur (Arhar), Lentil (Masur), Guar, Gram, and Mung
(d) Cash crops: Sugarcane, Cotton, Jute, and Mesta

At the crop group level, cash crops contributed the largest share (52.41%) in agro produce, followed by cereals (39.43%), oilseeds (4.82%), and pulses (3.34%) in 2019–2020. It is interesting to mention that cash crops have the smallest share (10.75%), and cereals had the largest share (57.29%) in the sown area in 2019–2020. However, at the individual crop level, sugarcane contributes the largest share (51.28%) in gross agro produce, followed by rice (17.08%), wheat (15.51%), and maize (4.13%). The sown area, production, and yield of principal crops during 2019–2020 in India have been summarized in Table 11.1.

Researchers have attempted to estimate agro residues generation in the country, and there has been significant variation in their findings. Hiloidhari et al. studied crop harvesting with 2011–2012 as the base year and estimated that 624.8 MT agro residues had been generated annually (Hiloidhari et al. 2014). In a similar study, Chandel & Sukumaran (the base year 2012–2013), Rajagopal et al. (the base year 2014–2015), and Ravindra et al. (the base year 2016–2017) estimated 656.6, 626.7, and 487.7 MT annual agro residue generation, respectively (Chandel and Sukumaran

2017; Rajagopal et al. 2018b; Ravindra et al. 2019). According to various estimates, cereal crop residues have the highest share (54.76–75.01%) in overall agro residues, followed by cash crops (17.01–32.51%), oilseeds (3.01–9.42%), and pulses (2.86–5.28%). Agro residues find applications as animal fodder, solid fuel for household cooking and heating, and feedstock for traditional rural industries such as rice mills, brick kilns, and potteries (Kishore et al. 2004; Agrawal and Verma 2020a). The left-over residues are accounted for as surplus residues. According to the estimates, India has a potential of 116.82–219.4 MT of surplus agro residues. Estimates of gross and surplus agro residues availability by various studies have been summarized in Table 11.2.

11.3 Agro Residues Valorization Through the Thermochemical Route

Agro residues are often considered waste in developing economies like India, and their eco-friendly and safe disposal is a significant challenge. However, agro residues are organic and have moderate (14–21 MJ/kg) calorific values, which offers promising energy generation potential (Anand et al. 2021). Agro residues are converted into useful fuel, heat, and electrical energy through several thermochemical and biochemical routes. In thermochemical conversion routes, whole biomass is heated in a controlled oxygen environment to convert it into modified energy forms. Thermochemical conversion technologies include direct combustion, gasification, and pyrolysis (Kargbo et al. 2021; Shen and Yoshikawa 2013; Goswami et al. 2021b). In the biochemical route, enzymes, bacteria, and engineered microorganisms convert biomass into drop-in fuels such as methane and ethanol (Jiang et al. 2019; Singh et al. 2016; Bhardwaj et al. 2021; Agrawal and Verma 2020b). Biochemical conversion technologies include anaerobic digestion and fermentation. Biogas is obtained through anaerobic digestion of agro residues. Sugar and starch crops such as sugar beet, sugarcane, wheat, and maize are subjected to fermentation for ethanol production. Routes for agro residues valorization for energy generation have been shown in Fig. 11.2.

Thermochemical conversion technologies such as direct combustion and gasification have been disseminated on larger scales for electricity generation. The product yield of biomass gasification and pyrolysis has been summarized in Table 11.3.

Agro residues valorization through the thermochemical conversion route has been discussed in the following section.

11.3.1 Direct Combustion

Agro residues have been used for cooking and heating since ancient. Currently, direct combustion of agro residues in boiler-steam turbine electricity generation

Table 11.2 Gross and surplus agro residues estimates in India

Crop category	Crops	Gross agro residues (MT)				Surplus agro residues (MT)			
		Hiloidhari et al. (2014)	Chandel and Sukumaran (2017)	Rajagopal et al. (2018a)	Ravindra et al. (2019)	Hiloidhari et al. (2014)	Chandel and Sukumaran (2017)	Rajagopal et al. (2018a)	Ravindra et al. (2019)
Cereal	Rice	154	151.55	164.68	165.82	43.5	9.92	43.5	50.23
	Wheat	131.1	135.59	123.76	141.44	28.4	11.1	28.4	30.37
	Maize	35.8	43.41	42.25	58.53 (All cereal except rice and wheat)	9	5.84	9	12.85 (All cereal except rice and wheat)
	Bajra	24.3	10.93	17.38		5.1	1.61	5.1	
	Jowar	17.6	10.56	14.12		3.5	1.08	9	
	Other nutri cereals	4.9	7.52	4.65		0.6	0.75	0.6	
Oilseeds	Rapeseed and mustard	12.7	16.06	13.55	14.63 (All oilseeds)	4.9	18.55 (All oilseeds)	4.9	3.50 (All oilseeds)
	Soybean	13.5	29.3	15.68		4.6		4.6	
	Sunflower	3.8	1.08	3.09		0.6		0.6	
	Groundnut	17	9.4	18.6		3		3	
	Other oilseeds	1.8	6	4.11		0.6		1.7	
Pulses	Lentil (Masur)	1.7	1.41	–	24.39 (All pulses)	0.3	6.25 (All pulses)	–	3.50 (All pulses)
	Gram	6.4	8.83	10.17		1.6		1.6	
	Tur (Arhar)	7.2	3.78	6.32		1.4		1.4	
	Other pulses	2.6	6.7	16.59		1.8		3	

Cash crops	Sugarcane	110.6	145.01	90.72	63.40	55.7	77.56	55.7	11.68
	Cotton	75.9	68.44	60.58	14.63	46.9	41.65	46.9	3.50
	Jute and Mesta	3.9	–	20.41	4.88	0.4	–	0.4	1.17

– data not reported

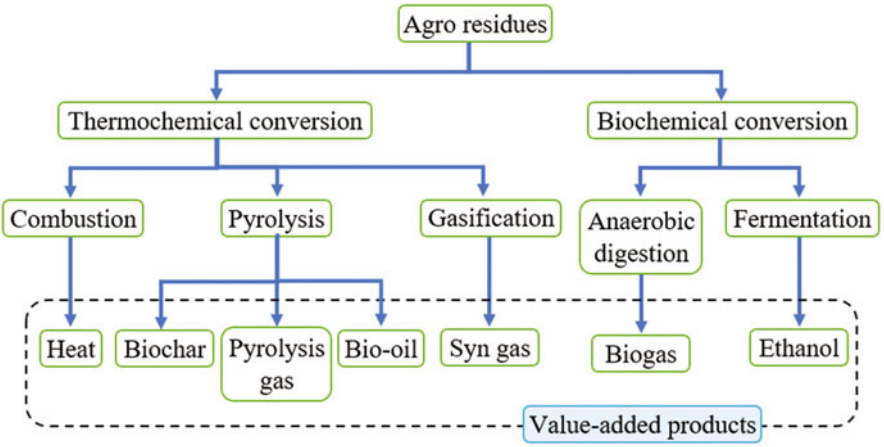

Fig. 11.2 Agro residues valorization for energy generation (Adopted from: Hossain 2017; Jiang et al. 2019)

Table 11.3 Product yields in gasification and pyrolysis process (Sources: AlQattan et al. 2018; Basu et al. 2011; Sakhiya et al. 2020)

Thermochemical process		Gasification	Pyrolysis		
			Fast/Flash	Intermediate	Slow
Process parameters	Temperature (°C)	700–1200	300–1000	300–450	300–600
	Heating rate (°C/min)	–	>300	>50	2–15
	Solid residence time	–	<10 s	10 min	>60 min
	Vapor residence time	Long	0.5–2 s	10–30 s	>30 s
	Biomass size	Briquette/ Chopped/Coarse	Powder/Finely ground	Coarse/ Chopped	Briquette/ Whole
Product yield (%)	Bio-oil	5–7	50–70	35–50	20–50
	Biochar	10–12	10–30	25–30	25–45
	Gas	80–85	15–20	20–30	20–50

systems is disseminated for large-scale electricity generation. Boiler-steam turbine electricity generation technology is similar to coal-based thermal power plants except for the boiler. It involves agro residue oxidation with excess air to produce heat in the conventional ranking cycle, which produces steam in a high-pressure boiler. The steam is then used to generate electricity using steam turbines. The exhaust of steam turbines could also be utilized for useful heating purposes called cogeneration. Cogeneration mainly finds its application in industries such as sugar mills in India. Modern combustion systems facilitate controlled biomass combustion. It minimizes emissions (such as CO, SO_2, NO_x, and particulate matter) caused by improper oxygen supply and incomplete combustion.

Agro residues have proximity to low and medium-grade coal in terms of carbon content, heating value, and ignition temperature. So, agro residues co-firing with coal can also be employed for electricity generation, and 36–44% electricity generation efficiency has been achieved (Basu et al. 2011). Although 20% biomass co-firing with coal is mandated in power plants, only 5–10% co-firing has been implemented. Agro residues—coal co-combustion offers higher electricity generation efficiency than agro residues alone, and agro residues ash captures sulfur. However, co-firing arises challenges such as high corrosion, slagging, and fouling due to higher alkali content in agro residues. Projected gradual phaseout of coal-based thermal power plants also minimizes the co-combustion prospects.

There is more than 40 GW installed facility for biomass-based electricity generation worldwide. These projects have been very successful across the globe, including countries in Europe, Africa, Asia, and Southeast Asia. In India, there are about 800 agro residues-based electricity generation plants. These plants are operational in Chhattisgarh, Madhya Pradesh, Gujarat, Rajasthan, and Tamil Nadu (Ministry of New and Renewable Energy 2021). Out of 10.17 GW total installed capacity in 2020, bagasse cogeneration has the largest share (74.36%), followed by biomass generation (18.05%) and non-bagasse cogeneration (7.59%). These plants use locally available agro residues for electricity production and deliver grid-quality electricity to nearby houses, industrial units, and utilities. Therefore, agro residues have a promising prospect for biomass-based electricity generation.

Ministry of New and Renewable Energy (MNRE) is implementing an agency to develop biomass-based electricity generation in India. According to MNRE estimates based on agro residue's annual availability volume, India has approximately 28 GW of electricity generation capacity derived from agro residues. However, only one-third of biomass-based electricity generation facility has been installed in India. Although year-round cultivation of cereals, oilseeds, pulses, and cash crops in India makes agro residues readily available, existing biomass-based electricity generation facilities suffer due to seasonal harvest, limited storage, supply-chain constraints, and mainstream financing. Supply-side constraints could be addressed by introducing modern technologies such as GIS-based demand-supply channel, mechanized agro residues collection, transportation, and handling. International standardization for environmental health and safety norms and public-private investment could spur new opportunities for the biomass-based electricity generation sector.

11.3.2 Pyrolysis

Pyrolysis is considered as most effective thermochemical conversion route for biomass valorization. Biomass pyrolysis produces biochar, bio-oil, and pyrolysis gas, i.e., products in all three states of matter. Biochar can be co-fired with coal in existing thermal power plant facilities, reducing coal dependency for electricity generation. Bio-oil can be refined and upgraded to drop-in fuel grade to be utilized

in the transportation sector. Pyrolysis gas can be captured and utilized for pre-heating and thus enhancing pyrolysis process efficiency.

Biomass undergoes various complex reactions during different stages of pyrolysis. These reactions include dehydration, hydrocarbon cracking, isomerization, aromatization, polymerization, and secondary deposition (Kargbo et al. 2021). These reactions are highly influenced by process parameters such as process temperature, heating rate, solid and vapor residence time, carrier gas type and its flow rate, catalyst, and others and influence product yield distribution. Slow heating (2–15 °C/min) at 300–600 °C and more than 60 min solid residence time yield good quality biochar. Feedstock in briquette/coarse form is considered for slow pyrolysis (Sakhiya et al. 2021). However, a high heating rate (<300 °C/min) with minimum vapor residence time at 300–1000 °C maximizes bio-oil yield. Feedstock in powdered form is considered for fast pyrolysis (Onay and Kockar 2003). The pyrolysis process with a moderate heating rate (20–50 °C/min) is intermediate pyrolysis. Intermediate pyrolysis produces a balanced quantity of biochar, bio-oil, and pyrolysis gas. Process parameters and product yield distribution for slow, fast, and intermediate pyrolysis have been summarized in Table 11.3.

Depending upon the application and required physicochemical properties, pyrolysis could be catalytic, microwave-assisted, additive-assisted, CO_2-assisted, and solar pyrolysis. Catalysts such as zeolite, Co-Mo-Z, Ni-CaO-C, Fe_2O_3, and Al_2O_3 are used to reduce operating temperature and impart selectivity in the products (Sakhiya et al. 2020). Microwave-assisted and catalytic pyrolysis produces bio-oil to extract olefins, aromatics, and other valuable chemicals. CO_2-assisted pyrolysis provides a reactive medium to enhance pyrolysis gas yield and reduce bio-oil yield. Solar pyrolysis uses solar energy as a power source and decreases dependency on grid electricity, which ultimately reduces CO_2 emissions. A schematic diagram of pyrolysis has been shown in Fig. 11.3.

11.3.3 Gasification

Gasification renders biomass valorization a clean and acceptable fuel in environmental norms. It typically operates at 700–1200 °C and involves the thermochemical conversion of biomass to medium or low calorific syngas with a restricted air supply (AlQattan et al. 2018; Thomson et al. 2020). CO, CO_2, CH_4, and H_2 are the main constituents of the syngas. The syngas is then used in combined cycle power generation systems for electricity generation. In suitably modified internal combustion engines coupled with generators, syngas can partially substitute fossil-based fuel (60–80%) in diesel and 100% in gas-fired spark engines. Electricity produced in a combined power cycle has higher efficiency and is more economical than electricity produced in steam turbines. Syngas could also be used for heating purposes in various boilers, furnaces, hot air generators, and dryers.

Generally, gasification involves drying, pyrolysis, partial oxidation, and gasification. Moisture is removed in the first stage between 100 and 200 °C. Then, thermal

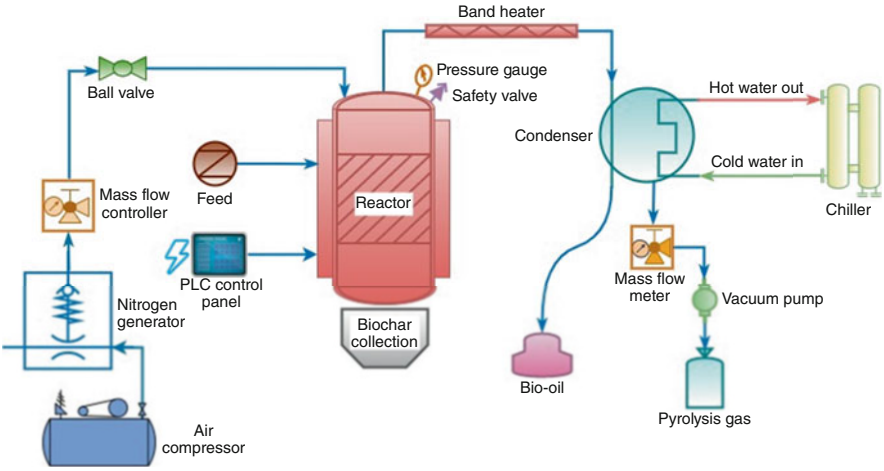

Fig. 11.3 Schematic diagram of batch pyrolysis setup

breakdown of lignin, cellulose, and hemicellulose of biomass took place during the pyrolysis stage to form biochar. In the third stage, partial oxidation of biomass and biochar occurs to produce CO, CO_2, and H_2O. Gasification becomes exothermic beyond 700 °C. CO_2 and steam act as gasifying agents and facilitate a reduction reaction with carbon and volatiles to produce syngas. A schematic diagram for the gasification setup has been shown in Fig. 11.4.

Process parameters such as feedstock composition and size, moisture and ash content, gasification temperature and pressure, gasifying agents, type of gasifier, and feedstock-air-steam equivalence ratio impact gasification efficiency. Microwave, supercritical, and plasma-assisted gasification improve gasification yield. Intense thermal plasma ionizes and catalyzes organic compounds in gas and biomass, respectively, into syngas. The supercritical water gasification process enriches H_2 and CH_4 content in syngas. At the same time, microwave-assisted gasification facilitates uniform temperature distribution and effective heating even in large biomass sizes.

11.4 Life Cycle Assessment of Thermochemical Conversion Route

Agro residues valorization through thermochemical conversion for energy generation has been well established. Biochar, bio-oil, and syngas obtained from thermochemical conversion can partially substitute fossil fuels for energy generation and transportation. Countering the increasing fossil fuel usage can reduce pressure on non-renewable fossil fuels and their environmental impacts. However, it is essential to quantify the real benefits of biochar, bio-oil, and syngas over conventional energy

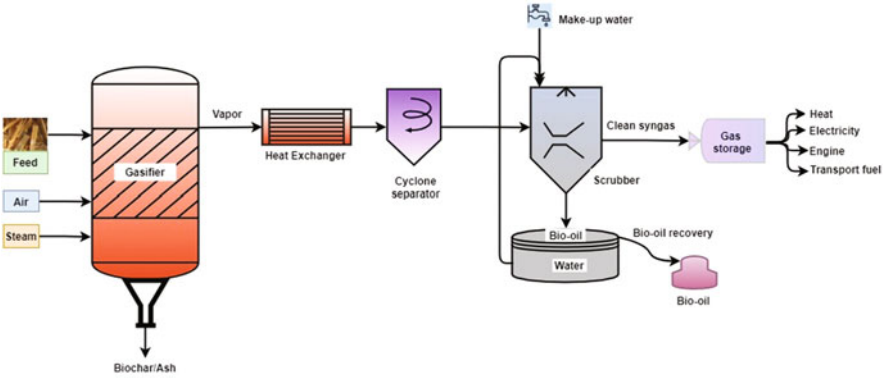

Fig. 11.4 Schematic diagram of gasification setup

sources such as coal and crude oil. Life cycle assessment (LCA) is identified as a practical, scientific, and robust tool for a comprehensive evaluation approach (Curran 2006; Kumar and Verma 2021). LCA can provide evidence-based insights for long-term strategic decisions-making for sustainable development. LCA procedure was standardized by International Organization for Standardization (ISO) under ISO14040 & ISO14044 in 2006 (Lee and Inaba 2004). There are four main stages of any life cycle study—goal and scope definition, life cycle inventory analysis, environmental impact assessment, and life cycle interpretation. In this section, environmental impacts of the entire production chain of biochar, bio-oil, and syngas have been deliberated to evaluate agro residues to biofuel pathways critically. It includes analyzing existing LCA studies and highlighting key methodological approaches and their findings.

11.4.1 Goal and Scope Definition

Goal and scope definition is the first stage of a life cycle study. Purpose, aim, and objectives incorporating functional unit and system boundary of the life cycle study are defined at this stage. The functional unit of a life cycle study is a quantified description of the performance standards of a product system. Generally, Joule (J) or kilo-Watthour (kWh) is chosen as the functional unit for bioenergy-related products. There are various phases and processes involved in a product manufacturing system. For instance, fuel production from agro residues includes crop cultivation, agro residues generation, fuel production, and fuel usage at the end of life. System boundaries are defined to include/exclude processes considered for estimating environmental impacts. The first phase of agro residue generation from crop cultivation is considered. In the second phase, agro residue valorization through thermochemical conversion routes and separation/up-gradation to drop-in fuel is considered. The

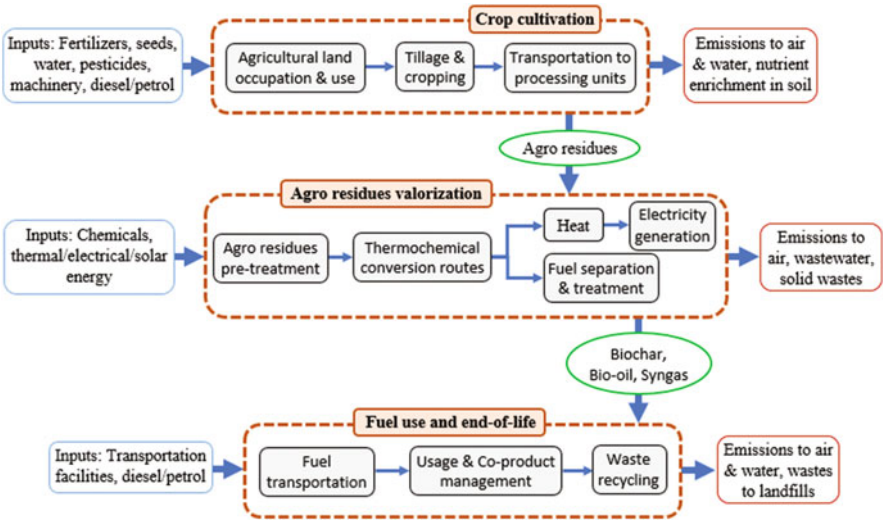

Fig. 11.5 Generalized system boundary for life cycle study of agro residue valorization to fuel and usage (Adopted from Osman et al. 2021)

third and last phase include the usage and end of life management phase. In this phase, biochar, bio-oil, and syngas are considered for electricity generation, transportation, and other purposes. It is interesting to note that most life cycle studies (about 90%) consider agro residue valorization for environmental impact assessment and life cycle interpretation (Osman et al. 2021). A generalized system boundary for agro residues valorization for fuel production has been shown in Fig. 11.5.

11.4.2 Life Cycle Inventory Analysis

All inputs and outputs such as raw materials and energy requirements, emissions in air and water, solid and liquid wastes, and others of the system boundary are quantified in this stage. It is worth mentioning that the larger the number of inputs, outputs, and processes in the system boundary, the greater the data would be required for inventory analysis. Ecoinvent, GREET, ASPEN Plus, Water footprint database, GLOBIOM model, and KTBL are popular databases globally used for life cycle inventory analysis (Herrmann and Moltesen 2015). Components of inventory analysis for selected life cycle studies on agro residues' thermochemical conversion have been listed in Table 11.4.

11.4.3 Environmental Impact Assessment

In this stage, critical environmental impacts are quantified under various categories. Impact categories are carefully chosen based on the functional unit, system boundary, system modeling, and aim and objectives of the life cycle study. For instance, the energy ratio is an important parameter to evaluate the usability of fuel sources. Therefore, most life cycle studies on fuel categorize energy ratio as energy return on investment (EROI), i.e., the ratio of output energy to input energy. Most studies reported EORI >1, which establishes agro residues-derived fuels as crucial energy sources (Reaño 2020). Environmental impact assessment is done at two points, namely—mid-point assessment and end-point assessment.

11.4.3.1 Mid-Point Assessment

Mid-point categories include those environmental impacts which cause immediate impact. These include global warming potential (GWP), abiotic depletion, ozone layer depletion, and others. GWP is generally expressed in kg-CO_2-equivalence within 100 years of time horizon. However, following infrastructure life span, temporal scales such as 20 years and 40 years are also considered. Aberilla et al. reported lower GHG emissions from thermochemical conversions than biochemical conversions (Aberilla et al. 2019).

Water depletion is expressed in m^3 and includes overall water usage during the entire thermochemical conversion process. About 84.6% of total water use occurs during crop production (Zhu et al. 2019). So, studies on water depletion recommended that water usage during energy crop cultivation and other processes be considered. However, water depletion due to agricultural crop cultivation could be dropped because agro residues are considered waste. Zhu et al. observed that biofuel production from cotton straw imparts a lower water footprint than fossil fuels but much greater than renewable energy sources such as wind, solar, and geothermal energy.

The land use category comprises the transformation of forest land for agricultural and other activities. It is expressed in m^2. Studies based on European Union and the United Kingdom concluded that land usage for energy crops could mitigate GHG emissions. However, biodiversity could also be lost. Studies on the combined effect of land use and water depletion reported that waste-derived biomass feedstocks could offer more sustainable energy than energy crops.

Abiotic depletion is related to the depletion of peat, clay, minerals, and fossil fuels. It is generally expressed in kg-Sb-equivalence. Ozone layer depletion potential is typically evaluated at 40 years time frame and expressed in kg-Trichlorofluoromethane-equivalence. Ecotoxicity potential is evaluated in three distinct categories to examine impacts on terrestrial, freshwater, and marine ecosystems. Photochemical oxidation documents those reactive substance emissions which are harmful to human health and the ecosystem. Eutrophication potential records the

Table 11.4 Components of inventory analysis for thermochemical conversion of agro residues

Region	Feedstock	Process	Functional unit	Product	LCA tool	Database	Reference
Southeast Asia	Rice and coconut residues	Combustion and gasification	1 kWh	Electricity	GaBi7.3	Ecoinvent3.1	Aberilla et al. (2019)
Southern Mexico	Sugarcane bagasse	Boiler	1 MJ	Bioenergy		Ecoinvent	Amezcua-Allieri et al. (2019)
EU27 & UK	Biomass from land		Biodiversity loss per hectare	Biofuel	GIS	GLOBIOM	Di Fulvio et al. (2019)
China	Cotton straw	Combustion	1 MJ	Electricity		Water footprint database	Zhu et al. (2019)
China	Biomass and water	Gasification	1 kg	Hydrogen			Wang et al. (2019)
Peru	Rice husk	Combustion	1 MJ	Electricity		Ecoinvent3.1	Quispe et al. (2019)
Southeast Asia	Rice husk	Gasification	1 kg	Bio-hydrogen	OpenLCA1.10	Ecoinvent3.5	Reaño (2020)
China	Crop residues	Slow pyrolysis	1 Tonne crop residue	Biofuel and biochar	GaBi8.7	PRC2019	Yang et al. (2021)
USA	Switchgrass and pine residues	Fast pyrolysis and combustion	1 MJ	Gasoline and diesel	GIS	GREET1.8	Lan et al. (2020)
USA	Forest residues	Fast and catalytic pyrolysis	1 kg	Biodiesel	ASPENplus & GaBi9	GaBi9 & LCA	Spatari et al. (2020)

release of excess nutrients. Human toxicity potential indexes two sub-categories—carcinogens and non-carcinogens, corresponding to potential harm by cancer-causing and non-cancer-causing chemical releases, respectively. Human toxicity is reported in 1,4 dichlorobenzene equivalence.

11.4.3.2 End-Point Assessment

Mid-point assessment results are aggregated into three end-point categories—human health, ecosystem quality, and resource depletion. The human health category is disability-adjusted life years due to environmental degradation. It accounts for life years lost due to ill health, permanent and temporary disabilities, and premature death. Ecosystem quality indicates the loss of biodiversity and local species due to global warming, ozone layer depletion, eutrophication, acidification, and ecotoxicity. Resource depletion is generally expressed in USD. It records raw materials and energy resource depletion and additional costs associated with future resource extractions such as minerals and fossil fuels.

11.4.4 Uncertainty and Sensitivity Analysis of Scenarios

Models in life cycle study are often simplified depictions of real-world complex systems. So, there are inherent uncertainties involved in the results of a life cycle study. These uncertainties arise from model approximations, spatial and temporal variability, intrinsic arbitrariness, statistical differences, expert disagreements, subjective conclusions, and linguistic shortcomings.

Agro residues thermochemical conversion processes with the least environmental footprint are identified through sensitivity analysis. Identified processes become a part of future research and development to minimize environmental impact. Therefore, uncertainty and scenario analysis of various scenarios are essential for robust and responsible decision-making.

11.4.5 Interpretation of Results

Interpretation of results is the last stage of a life cycle study. It comprises conclusions and recommendations based on identified thermochemical conversion processes/phases to improve the environmental performance of agro residues-based fuel systems. The outcome of this stage is communicated to the stakeholders for informed decision-making. Results and findings of selected life cycle studies have been summarized in Table 11.5.

Various life cycle studies have established that biochar, bio-oil, and syngas production from pyrolysis and gasification is a reliable and sustainable alternative

Table 11.5 Results and findings of selected life cycle studies

References	Environmental impact categories	Results
Aberilla et al. (2019)	GWP (20 years), ozone layer depletion, and human toxicity (carcinogen)	GWP: −30 to 30 kg CO_2 eq Ozone layer depletion: −1 μg CFC-11 eq Human toxicity (carcinogens): 72–182 g 1,4 DB eq
Amezcua-Allieri et al. (2019)	Eutrophication, freshwater aquatic, marine and terrestrial ecotoxicity, acidification, ozone layer depletion, and abiotic resource depletion	Results were reported in terms of the potential environmental impact index (PEI), which is the total of all impact categories For sugarcane bagasse use: 2528 PEI/GJ For fossil fuel use: 20,200 PEI/GJ
Di Fulvio et al. (2019)	Biodiversity loss	Energy crop cultivation can mitigate climate change. However, it can also negatively impact biodiversity due to habitat loss of native species
Zhu et al. (2019)	Water footprint in L/MJ bioenergy	For combustion: 11.708 L/MJ, lower than electricity generation from bio-oil but much higher than renewable energy sources like geothermal, solar and wind power
Wang et al. (2019)	GWP	An increase in gasification temperature and pressure could decrease GWP
Quispe et al. (2019)	GWP (100 years), water depletion, eutrophication, and acidification potential	457 MJ energy derived from 9.5 T/ha rice husk yield at 0.7 dryer efficiency. Following were the impacts: GWP: 1.96 kg CO_2 eq Acidification potential: 50.3 g SO_2 eq Eutrophication potential: 12.5 g PO_4 eq Water depletion potential: 2.3 m^3 For coal GWP: 61 kg CO_2 eq; Acidification potential: 462 g SO_2 eq; Eutrophication potential: 63 g PO_4 eq; Water depletion potential: 23 m^3 Replacing coal with rice husk could annually mitigate 708,540 T CO_2 eq
Reaño (2020)	EROI, GWP (100 years), terrestrial ecotoxicity, eutrophication, and acidification potential	EROI: 1.25 GWP: 46 kg CO_2 eq Acidification potential: 75 g SO_2 eq Eutrophication potential: 350 g PO_4 eq Terrestrial ecotoxicity potential: 2.5 mg 1,4 DB eq

(continued)

Table 11.5 (continued)

References	Environmental impact categories	Results
Yang et al. (2021)	GWP (100 years) and abiotic resource depletion	Biochar system could sequestrate 500 GT at the country level, which is 4.5% of annual GHG emissions from China Abiotic depletion potential: $4.5*10^7$ kg Sb eq/kg biochar
Lan et al. (2020)	GWP (100 years) and primary energy consumption	Primary energy consumption & GWP for 1 MJ of gasoline and biodiesel production from switchgrass and pine residues were 0.7 & 1.1 MJ, and 43.2 & 76.6 g CO_2 eq, respectively
Spatari et al. (2020)	GWP (100 years)	GWP for catalytic pyrolysis: −80 g CO_2 eq/kg biodiesel GWP for fast pyrolysis: 20 g CO_2 eq/kg biodiesel

to substitute/partially replace fossil fuels in the present energy systems. Agro residues-derived fuels have also shown tremendous potential for mitigating resource depletion such as water, impacts due to land use, and GHG emissions. Therefore, agroresidues-derived energy systems could be established.

11.5 Conclusion

Agro residues are often considered waste in developing countries like India. Thermochemical and biochemical conversion of agro residues for energy application is in line with the zero-waste hierarchy for waste management. Even if fuel production from the thermochemical conversion route is more energy-intensive than the biochemical route, the thermochemical conversion route is preferred for large-scale fuel production. It is because of lengthy biochemical routes. Life cycle study on thermochemical conversion of agro residues for fuel application has established lower GHG emissions and environmental footprints than conventional fossil fuels. Readily available agro residues are cheaper than conventional energy sources. Therefore, thermochemical conversion of agro residues for fuel application can be an economical, reliable, and sustainable alternative in the long term.

Competing Interests The authors declare that they have no competing interests.

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