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Recent Advances in Bioethanol Production from Lignocelluloses: A comprehensive focus on enzyme engineering and designer biocatalysts

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Abstract

Most of the countries have implemented their biofuel policy programme for sustainable development. However, there is a limitation of availability of sufficient quantity of ethanol to reduce the carbon footprints due to bottlenecks in the present technology to convert multiple feedstocks including lignocelluloses. Whereas first generation ethanol made from edible sugar resources, mainly sugarcane and its molasses, and starch grains, primarily corn and wheat, is an established process, lignocellulosic ethanol production has several process bottlenecks, affecting process yields and economics. Sustainable and cost-effective technology is need of the hour to mitigate the demand and supply of lignocellulosic ethanol round the year at affordable price. This review article describes the recent advances in the pretreatment of lignocellulosic biomass, with the focus on the use of green solvents including ionic liquids and deep eutectic solvents, followed by enzymatic saccharification using auxiliary proteins for the efficient saccharification of pretreated biomass. Different techniques for strain improvement strategies to develop hyper-producing deregulated lignocellulolytic strains have also been discussed and compared. The advanced techniques for fermentation of mixed sugars present in lignocellulosic hydrolysates for maximizing bioethanol production have been summarized with emphasis on pathway and transporters engineering for xylose assimilation. Further, the integration of different steps is suggested for efficient biomass utilization and improved ethanol yields and productivity.

Keywords: Pretreatment; enzymatic saccharification; auxiliary proteins; pathway engineering; transporter engineering; integrated fermentation

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Abbreviations:

[BMIM][$MeSO_3$]	1-butyl-3-methylimidazolium methanesulfonate
[EMIM][DEP]	1-ethyl-3-methylimidazolium diethyl phosphate
[emim][OAc]	1-ethyl-3-methylimidazolium acetate
[TBA][OH]	Tetrabutylammonium hydroxide
2G	Second generation
AA	Auxiliary activity
AFase	α-L-arabinofuranosidases
AXEs	Acetyl xylan esterases
bglR	β-glucosidase regulatory gene
CADO	Consolidated alcohol dehydration and oligomerization
CAZymes	Carbohydrate active enzymes
CBH	Cellobiohydrolases (Exoglucanases)
CBMs	Carbohydrate binding modules
CBP	Consolidated bioprocessing
CCR	Carbon catabolite repressor
CDH	Cellobiose dehydrogenases
CEs	Carbohydrate esterases
CEs	Carbohydrate esterases
CFER	Cell-free extract reaction
CFP	Cotton filter powder
CH_4	Methane
ChCl-G	Choline chloride-glycerol
ChCl-LA	Choline chloride-lactic acid
ChCl-U	Choline chloride-urea

CMC	Carboxymethyl cellulose
CO_2	Carbon dioxide
COVID-19	Corona virus disease
cre1	Carbon repressor gene
CRISPR-Cas	Clustered Regularly Interspaced Short Palindromic Repeats
DCR	Dirty cotton residue
DES	Deep eutectic solvents
EG	Endoglucanases
EMS	Ethanomethane sulphate
EP-PCR	Error-prone PCR
FAD	Flavin-adenine dinucleotide
FAEs	Feruloyl esterases
FGRFS	Federal government renewable fuel standards
GAX	Glucuronoarabinoxylan
GHGs	Greenhouse gases
GHs	Glycosyl hydrolases
gls 2α	glucosidase IIα subunit of gene
gpd	Glyceraldehyde-3-phosphate dehydrogenase gene
gTME	Global transcription machinery engineering
HMF	5-hydroxymethylfurfural
ILs	Ionic liquids
LCB	Lignocellulosic biomass
LiP	Lignin peroxidases
LMCOs	Laccase like multicopper oxidases
LPMOs	Lytic polysaccharide monooxygenases

MDF	Max-min driving force
MNNG	N-methyl-N'nitro-N-nitrosoguanidine
MnP	Manganese peroxidases
N_2O	Nitrous oxide
OPEFB	Oil palm empty fruit bunch
PASC	Phosphoric acid swollen cellulose
PCTR	Pilot-scale continuous tubular reactor
PEG	Polyethylene glycol
PPE	Personal protective equipment
rDNA	Recombinant DNA
RNAi	RNA interference
SHF	Separate hydrolysis and fermentation
SiRNAs	Short-interference RNAs
SSCF	Simultaneous saccharification and co-fermentation
SSF	Simultaneous saccharification and fermentation
$t_{1/2}$	Half-life
VP	Versatile peroxidases
WHO	World Health Organization
XDH	Xylitol dehydrogenase
XI	Xylose isomerase
XK	Xylulokinase
XR	Xylose reductase
βG	β-glucosidases

1. Introduction

The ever increasing demand of energy due to rapid increase of global population, industrialization, and geopolitical factors have called for the search of alternative and carbon neutral sources of gasoline (Chandel et al., 2020; Souza et al., 2017). The primary sources of energy in the current decade are non-renewable fossil fuels, oil, natural gas, and coal. However, these energy sources are inadequate to fulfil the needs of enormously rising population due to their limited availability, resulting in energy crisis for the future generation. Moreover, these sources also contribute to climatic issues such as global warming and environmental pollution by releasing greenhouse gases (GHGs) like carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), and chlorofluorocarbons (Kiran et al., 2014). Several social, economic, and environmental issues such as burgeoning petroleum prices, finite nature of fossil fuels, increasing environmental concerns towards the release of GHGs, and health and safety concerns encourage the government and non-government agencies to find environmentally friendly, renewable, sustainable, and alternate energy sources for transportation, heating, and electricity generation (Nikolić et al., 2016).

Other than fulfilling the energy demand of rapidly growing world's population, ethanol is also used to combat pandemic diseases including corona virus disease (COVID-19). World Health Organization (WHO) has recommended the use of disinfectant containing alcohols like ethanol and isopropanol for the adequate inactivation of coronavirus which is published in the Journal of Emerging Infectious Diseases by researchers from Ruhr-Universitat Bochum (RUB) in Germany (https://www.thehindu.com/news/international/who-recommended-disinfectants-effective-against-novel-coronavirus-study/article31386598.ece) (Kratzel et al., 2020).

Lignocellulosic biomass (LCB) is used as feedstock for second generation (2G) biofuels, preferably for bioethanol production due to wide availability, easy and cheaper

accessibility of LCB. Renewable LCB is used in production of 2G biofuels, therefore, it does not lead to problems of food scarcity (Branco et al., 2019; Kuila et al., 2016). LCB derived liquid biofuels, as one of the major renewable energy sources, can cover approximately 40% of the total energy consumption of world due to their technical feasibility, easy availability, and wide acceptability (Gielen et al., 2019; Meher et al., 2006).

LCB forms a major fraction of plant matter, and widely available as largely generated agricultural residues, forestry residues, industrial wastes (e.g. sugar, and paper and pulp industries, etc.), domestic/municipal wastes, etc. Different agricultural wastes which are available in plenty for bioethanol production are banana plant waste (Ingale et al., 2014; Jahid et al., 2018; Khaliq et al., 2020), barley straw (Lara-Serrano et al., 2018), corn stover (Dhiman et al., 2017; Zakir et al., 2016), cotton stalk (Nikolić et al., 2016), and sugarcane bagasse, straw (Cheng et al., 2019; Wong and Sanggari, 2014; Zakir et al., 2016) have been utilized previously for bioethanol production.

Three major steps are involved in lignocellulosic biomass conversion to fuel ethanol, *viz*., pretreatment, saccharification, fermentation and distillation. Lignocelluloses are composed of complex polysaccharides, which are highly resistant to degradation by chemical and enzymatic methods, due to closed packing within recalcitrant lignin structure (Haldar and Purkait, 2020). Hence, in spite of high availability of cost-effective raw material, i.e., LCB, the production of fuel ethanol and high value-based products with high yield and productivity is still a challenge (Kumar et al., 2008). The pretreatment process is performed to remove or redistribute the lignin, to reduce the cellulose crystallinity, and to increase the porosity significantly (McMillan, 1994). Subsequent saccharification or hydrolysis is done by acids or enzymes to hydrolyse the polymeric cellulose and hemicellulose into fermentable monomeric sugars (hexoses and pentoses) after pretreatment of LCB. Enzymatic hydrolysis is preferred over acid hydrolysis due to lower energy requirement and reduced by-products formation.

Nevertheless, enzymatic hydrolysis is influenced by several factors such as accessible surface area, cellulose crystallinity and degree of polymerization, lignin content, and enzymes synergy and effectiveness (Cheng et al., 2019; Kucharska et al., 2020; Lugani and Sooch, 2018; Myat and Ryu, 2016). After pretreatment and hydrolysis, fermentation of monomeric sugars is accomplished via microbial action to produce ethanol. The selection of microorganism for industrial bioethanol production depends upon its ability to utilize wide range of substrates, resistance against various inhibitory products, tolerance to high sugar and ethanol concentrations (Hans et al., 2019). The yield and productivity of ethanol is very less with wild microbial strains, hence, developing genetically modified microbial strains which can meet these requirements at industrial scale is the primary focus nowadays. The aim of current review is to cover advanced approaches for the pretreatment of lignocellulosic biomass, enzymatic saccharification, development of modified microbial strains to improve bioethanol yield, and different action mechanisms for bioethanol production using wild and genetically modified strains. It also provides a summary of various integration approaches used for fermentative production of bioethanol. The novelty of the present review lies by covering the advancement in the process from feedstock to product. The past review articles published in last five years are summarized in Table 1.

2. Pretreatment

Pretreatment is a necessary step to unwind the complex structure of LCB composed of mainly cellulose, hemicellulose, and lignin (Kassaye et al., 2017). LCB requires suitable pretreatment method for breaking down/redistribute the recalcitrant lignin structure leading to the accessibility of polysaccharides towards hydrolytic enzymes for hydrolysis into monosaccharides. An efficient pretreatment method makes the hydrolysis process more efficient with improved yields of monomeric sugars, reduced degradation of carbohydrates, and reduced formation of inhibitory by-products (Procentese et al., 2017). Therefore, finding

an effective biomass pretreatment is the most important challenge in second generation biofuels production as it should be convenient, environment friendly, and economically feasible (Ravindran et al., 2018). A variety of pretreatment methods have been developed for LCB conversion over the past few decades; however, there is no single strategy available so far that could be suitable for all types of feedstocks.

2.1. Conventional pretreatment approaches for lignocellulosic biomass

The most commonly used pretreatment technologies for LCB conversion include physical, (thermo) chemical, physico-chemical, and biological methods (Baruah et al., 2018; Behera et al., 2014; Gabhane et al., 2020; Hans et al., 2020; Kumar and Sharma, 2017). These are extensively studied methods but are associated with a variety of limitations such as lower yield, high processing cost, and negative environmental impacts, therefore, more efficient green technologies are being explored continuously to overcome these challenges (Capolupo and Faraco, 2016). Fig. 1 shows the different pretreatment approaches, and their pros and cons.

2.2. Green pretreatment approaches

Recently, the "Green Chemistry" concept has gained attention with a possible solution to the challenges of negative environmental impacts of conventionally used pretreatment methods involving the use of hazardous chemicals. Ionic liquids (ILs) and deep eutectic solvents (DES) based pretreatments are being developed as an attractive alternative to the conventional pretreatment strategies for lignocellulosic materials due to their ability to pretreat and selectively dissolve the constituents of biomass in non-hazardous manner.

2.2.1. Ionic liquid pretreatment

ILs are known as "green solvents" due to higher thermal and chemical stability, low vapour pressure, and non-flammable character (Wahlström and Suurnäkki, 2015; Wu et al., 2014). ILs are liquids composed of ions with strong electrostatic bonding, which makes ILs

as less volatile, and electrochemically stable to be applicable to dissolve and convert the biomass into different types of products (Socha et al., 2014). Moreover, the characteristic of ILs may be designed by altering the combination of cations and anions. The cations of ILs are organic, while anions may be inorganic or organic (Brandt et al., 2011).

Many studies have revealed that ILs pretreated LCB exhibited the increased surface area with reduced lignin content and cellulose crystallinity (Kassaye et al., 2017; Li et al., 2010; Wu et al., 2011). The developments made in the utilization and characterization of LCB using ILs over the past decade are reviewed and represented in Table 2. Xia et al. (2014) evaluated that switchgrass (*Panicum virgatum*) pretreated with 1-butyl-3-methylimidazolium methanesulfonate ([BMIM][$MeSO_3$]) at 110°C for 4 h resulted in much higher enzymatic hydrolysis glucose yields. Financie et al. (2016) observed that oil palm frond biomass (OPFB) pretreated with 1-ethyl-3-methylimidazolium diethyl phosphate [EMIM][DEP] at 110°C for 1 h showed the enhancement in cellulose content from 45.7% to 68.5.1% while reduction of 8.5% and 12.1% was observed in lignin and hemicellulose contents, respectively. Wang et al. (2018) investigated an ultrasound-assisted aqueous IL tetrabutylammonium hydroxide ([TBA][OH]) pretreatment approach for eucalyptus. The pretreated sample showed a marked enhancement in the initial enzymatic rate of cellulose (79.39 mg/g/h) as compared to untreated sample (17.63 mg/g/h). Recently, Fockink et al. (2020) observed the effect of 1-ethyl-3-methylimidazolium acetate [emim][OAc] on pretreatment of dirty cotton residue (DCR) and cotton filter powder (CFP) at 140°C for 2 h. DCR yielded high glucose yield of 78%, xylose yield of 94.9% and delignification levels of 45.5%, while CFP provided glucose yield of 75.8%, xylose yield of 95.7%, and 16% of delignification.

Although as reported in the studies, ILs pretreatment approaches are environment friendly but the high cost of these strategies may limit their use in large scale biorefinery (Hou et al., 2013).

2.2.2. Deep Eutectic Solvent Pretreatment

Abbott et al. (2003) attracted the attention towards a nascent class of green and designer solvents known as deep eutectic solvent (DES) as an alternative to ILs. DESs are a mixture of organic compounds having a hydrogen-bond acceptor, typically a quaternary ammonium halide salt and a hydrogen-bond donor such as amino acids, urea, amines, carboxylic acid, or carbohydrates (Francisco et al., 2012; Zhang et al., 2016). DESs are promising alternative to ILs in terms of cost, nontoxicity, and biodegradable features, and have attracted great attention in recent years. The use of DESs has been explored as extraction solvents (García et al., 2016; Jenkin et al., 2016), reaction media, and electrolytes (Alonso et al., 2016; Jhong et al., 2009). DESs have been used extensively in recent years in the pretreatment of LCB for achieving high hydrolysis and fermentation yields as given in Table 3.

Several studies have reported DES as a reasonable reaction media for enzymatic reactions as compared to conventional organic media (Erbeldinger et al., 1998; Gill and Vulfson, 1994). Many researchers have reported that DESs are promising solvents for dissolving most of the lignin from LCB (Kandanelli et al., 2018; Kim et al., 2018). Francisco et al. (2012) observed that choline chloride-lactic acid (ChCl-LA) in a molar ratio of 1:10 at 60°C for 24 h dissolved a high amount of lignin (11.82%) from wheat straw, while cellulose was intact. An et al. (2015) studied the pretreatment of grasses with cholinium-arginate, and observed >69% of lignin extraction. Pretreatment of rice straw with ChCl-LA was reported in a study to dissolve 68 ± 4 mg/g of lignin (Kumar et al., 2016). Solubilization of lignin and hemicellulose with more than 90% digestibility of cellulose have been reported with the pretreatment of corn stover and corncobs using DES (Hou et al., 2017; Xu et al., 2016; Zhang

et al., 2016). Loow et al. (2018) reported that pretreatment of oil palm empty fruit bunch (OPEFB) with a mixture of choline chloride-urea (ChCl-U) (1:2) (120 °C, 4 h) and 0.4 mol/L $CuCl_2$ (120 °C, 30 min) resulted in high delignification, and a xylose concentration of 14.76 g/L in hydrolysate (Loow et al., 2018). Another report showed similar results for DES-pretreated food wastes, such as apple residues, potato peels, and brewer's spent grains for pretreatment at 150°C for 16 h (Procentese et al., 2018). In this study, ChCl-U pretreatment lowered the energy requirement about 28% as compared to NaOH pretreatment.

The effect of three different DESs, ChCl-LA, ChCl-U, and choline chloride-glycerol (ChCl-G), was studied on pretreatment of OPEFB at 120°C for 3 h, with the solid-to-liquid ratio of 1:10 (w/v), and compared with acid and alkaline solvents. ChCl-LA (1:2) showed the highest reducing sugars yield (20.7%) and was found more effective than acid and alkaline solvents in preventing sugars loss and exposing its cellulose fraction to enzymatic saccharification (Thi and Lee, 2019).

3. Enzymatic saccharification of lignocellulosic biomass

Conversion of LCB into pentose/hexose sugars with industrially desired yield is one of the major bottlenecks in the production of 2G biofuels since there are several challenges associated with achieving high process efficacies. Although acid hydrolysis is the most convenient and widely employed method for hydrolysis of polysaccharides into monomers via breakdown of hydrogen bonding between cellulose chains and converting its crystalline form into entirely amorphous state. However, its corrosive nature, degradation of released sugars, difficulties in sugar and acid recovery from the mixture, high energy requirements, formation of fermentation inhibitors, and several environment related issues increase the process cost, and hence, limit its use (Al-Battashi et al., 2019; Binod et al., 2011; Harmsen et al., 2010; Sun and Cheng, 2002). On the other hand, enzymatic saccharification of LCB is a

suitable alternative as it is mild and eco-friendly approach with lower energy requirements (Al-Battashi et al., 2019; Rai et al., 2016b; Ummalyma et al., 2019).

The enzymes required for efficient deconstruction of polysaccharides into monomeric sugars constitute modular and non-modular glycosyl hydrolases (GHs) comprising of cellulases and hemicellulases, carbohydrate esterases (CEs), and auxiliary activity (AA) proteins (Chylenski et al., 2017; Ezeilo et al., 2017). The techno-economic analysis of the enzyme mediated 2G biofuel production processes remain a much debated topic owing to limited information of enzyme costs available in public domains. The enzyme costs reported in literature accounting for overall 2G ethanol production depends upon several factors apart from the cost of the enzymes. There are several reports in literature which vary significantly in their estimates of the enzyme costs (2G ethanol production) including USD 0.10/gal (Aden and Foust, 2009), 0.30/gal (Lynd et al., 2008), 0.32/gal (Dutta et al., 2010) and 0.40/gal (Kazi et al., 2010). However, such an inconsistency in the cost estimation hinders the robust techno-economic analysis of 2G ethanol production.

3.1. Hydrolytic enzymes

3.1.1. Glycosyl hydrolases (GHs)

GHs mediate the cleavage of glycosidic bonds that connect two or more sugars or one sugar and one non-sugar moiety within oligosaccharides (Ezeilo et al., 2017; Sathya and Khan, 2014). A total of 115 GH families comprising modular and non-modular cellulases and hemicellulases have been identified till date. These families of enzymes have been classified on the basis of their amino acid sequences and modes of action (Ezeilo et al., 2017; Rai et al., 2016a; Rai et al., 2016b). The catalytic activity of GH family enzymes is achieved either through inversion or retention mechanisms resulting into products with stereochemistry opposite and identical to the substrate, respectively (Davies and Henrissat, 1995; Jayasekara and Ratnayake, 2019). The mode of action of lignocellulolytic enzymes is shown in Fig. 2.

Cellulases are the dynamic constituents of GH enzymes that hydrolyze the most dominant polysaccharide on the earth, cellulose, to yield hexose sugars (mainly glucose) (Bayer et al., 1998; Mandels and Weber, 1969; Patel et al., 2019). The obtained sugars can then be fermented into ethanol, and additional valued products (Rai et al., 2016b). Cellulases include three main hydrolytic enzymes: endoglucanases (E.C.3.2.1.4; EG), exoglucanases (E.C.3.2.1.174 and E.C.3.2.1.91; cellobiohydrolases (CBH)), and β-glucosidases (E.C.3.2.1.21; βG). Endoglucanases (EGs) have been reported from GH families 5, 6, 7, 8, 9, 12, 44, 45, 48, 51, 74, and 124, and act randomly on β-1,4 glycosidic bonds of amorphous cellulose (Ezeilo et al., 2017; Sweeney and Xu, 2012). Among the identified families, EGs belonging to GH 6, 9, and 48 follow inversion mechanism, whereas, GH 5 and act through retention mechanism (Mingardon et al., 2007; Miotto et al., 2014). The catalytic action of these enzymes yield long-chain oligomers (with varying degrees of polymerization) possessing reducing and non-reducing ends which are processed by exoglucanases to form cellobiose (cellobiohydrolase, CBH) and/or glucose (β-glucosidases) in a processive manner (Ezeilo et al., 2017; Sweeney and Xu, 2012). Cellobiohydrolases belonging to GH families 5, 6, 7, 9, 48 and 74, act either from reducing (CBH I) or non-reducing (CBH II) ends of cellulose to produce short-chain oligosaccharides (Poidevin et al., 2013). Finally, βGs catalyze the hydrolysis of cellobiose and short cellodextrins yielding glucose as the final product (Ezeilo et al., 2017; Patel et al., 2019; Poidevin et al., 2013). βG enzymes are broadly classified as members of GH family 1, 3, 5, 9, 30, and 116; however, majority of the βG enzymes reported from fungi belong to the GH3 family (Singhania et al., 2013).

Hemicellulases, another group of glycosyl hydrolases, mediate depolymerization of hemicellulosic fraction of LCB (Ezeilo et al., 2017). Owing to its heterogenous structure, hemicellulose degradation requires a large suite of enzymes comprising endoxylanase (E.C.3.2.1.8), β-xylosidase (E.C.3.2.1.37), α-arabinofuranosidase (E.C.3.2.1.55), α-

glucuronidase (E.C.3.2.1.139), acetyl xylan esterase (E.C.3.1.1.72), arabinase (E.C.3.2.1.99), and feruloyl xylan esterase (E.C.3.1.1.73) (Bhattacharya et al., 2015; Ezeilo et al., 2017; Juturu and Wu, 2013). Among diverse hemicellulases, endoxylanases and β-xylosidases, collectively known as xylanases, are the most extensively studied enzymes. Endoxylanases have been identified primarily from GH families 5, 8, 10, 11 and 43, and are known to cleave β-xylosidic linkages that hold 2-D-xylopyranosyl residues together in the xylan backbone (Bhattacharya et al., 2015; Ezeilo et al., 2017; Lombard et al., 2014). Endoxylanases belonging to GH family 8 and 43 operate through the mechanism of inversion, whereas, those which belong to GH 5, 10, and 11 operate via retention mechanism. β-xylosidase, another essential component of hemicellulases, hydrolyze xylobiose and other short-chain xylo-oligosaccharides by attacking their β-1,4 linkages. Majority of the identified β-xylosidases have been reported from GH families 3, 39, 43, 52, and 54 (Bhattacharya et al., 2015; Knob and Carmona, 2009; Shallom and Shoham, 2003). Both inversion (GH 43) and retention mechanism (GH 3 and 54) of actions can be observed in β-xylosidases (Knob and Carmona, 2009; Shallom and Shoham, 2003). Further, α-L-arabinofuranosidases (AFase) and α-L-arabinases (collectively belonging to GH 3, 43, 51, 54, and 62) constitute another class of hemicellulases that is important for removal of arabinose residues from xylan backbone, which could have a synergistic effect on xylan hydrolysis. The arabinose substituted xylose residues inhibit the hydrolysis of glycosidic bonds in xylan backbone, and synergistic action of endoxylanases with these enzymes relieves this inhibition (Numan and Bhosle, 2006).

β-mannanases (GH 5 and 26) and β-mannosidases (GH 1, 2, and 5) degrade hemicelluloses made up of mannans into short manno-oligomers and subsequently into monomeric mannose (Shallom and Shoham, 2003).

3.1.2. Carbohydrate esterases (CEs)

Carbohydrate esterases formulate a distinct class of hydrolytic enzymes that are involved in the removal of ester flags from carbohydrates (Cantarel et al., 2009; Nakamura et al., 2017). These enzymes have been classified into 16 CE families ranging from CE1 to CE16; however, CE family 10 has been abolished since most of the members corresponding to this family were found to be active against non-carbohydrate substrates (Nakamura et al., 2017). There are diverse biotechnological applications assigned to CE proteins where the majority of the enzymes catalyze the elimination of ester-based alterations form mono, oligo, and polysaccharides. Therefore, CE mediated removal of acetylated moieties of polysaccharides could hasten up degradation of carbohydrates by facilitating GH proteins in accessing their target sites within biomass. The number of CEs analysed for their enzymatic function is very low (0.6%) with acetyl xylan esterases (AXEs) and feruloyl esterases (FAEs) being the most studied enzymes (Nakamura et al., 2017; Ulaganathan et al., 2015). Both AXEs and FAEs are the hemicellulolytic esterases where former hydrolyse acetyl substitutions on xylose fractions, and the later mediates hydrolysis of ester linkages holding ferulic acid and arabinose substituents together (Nakamura et al., 2017; Ulaganathan et al., 2015).

3.1.3. Auxiliary activity (AA) proteins

The conventional hydrolytic model for degradation of lignocelluloses has been updated with the discovery of a novel class of oxidative enzymes. These enzymes are capable of triggering the cleavage of glycosidic bonds within the glucose polymers through oxidative route and are referred to as auxiliary activity proteins (Ezeilo et al., 2017; Filiatrault-Chastel et al., 2019). The AA category encompasses a large class of carbohydrate active enzymes (CAZymes) that assist GH and CE enzymes acting on carbohydrates in the LCB (Ezeilo et al., 2017; Levasseur et al., 2013). Currently, AA category constitutes 9 families of lignin degrading enzymes and 6 families of lytic polysaccharide monooxygenases (LPMOs)

(Levasseur et al., 2013). Different AA families involved in the degradation of LCB are briefly described below.

AA1 family circumscribes multicopper oxidases with potential to utilize diphenols related compounds and oxygen as electron donor and acceptor, respectively (Levasseur et al., 2013). Laccases (EC 1.10.3.2), form a subfamily within AA1 family, is known to degrade LCB either by attacking lignin directly or eliminating inhibitors produced during pretreatment (Levasseur et al., 2013; Rai, R. et al., 2019). Laccase like multicopper oxidases (LMCOs; EC 1.10.3.2) constitutes another subfamily of AA1 proteins which shows involvement in delignification (Berni et al., 2019; Levasseur et al., 2013).

AA2 family encompasses another group of lignin modifying enzymes, also known as class II peroxidases (Levasseur et al., 2013). Lignin peroxidases (LiP; EC 1.11.1.14), manganese peroxidases (MnP; EC 1.11.1.13), and versatile peroxidases (VP; EC1.11.1.16) are the main enzymes of AA2 family where LiPs mediate oxidation of several aromatic phenolic compounds and a variety of non-phenolic lignin model compounds. On the other hand, VPs act as hybrid models of both LiPs and MnPs which consolidate their catalytic properties and oxidize phenolic, non-phenolic, and Mn^{2+} substrates (Janusz et al., 2017; Levasseur et al., 2013).

AA3 family comprises flavoproteins having flavin-adenine dinucleotide (FAD) binding domain. Cellobiose dehydrogenases (CDH; EC 1.1.99.18), the secreted hemoflavoenzymes produced by lignocellulolytic fungi under cellulolytic culture conditions, constitute the major proportion of this family (Bodenheimer et al., 2018; Levasseur et al., 2013). Another family of CAZymes, AA8 contain CDH enzymes that have cytochrome domains of spectral class b in their structural organization (Levasseur et al., 2013; Ma et al., 2017). Cellobiose dehydrogenases bind cellulose to mediate oxidation of cellodextrins, maltodextrins, and

lactose; and inhibit repolymerization of cellulose post cleavage (Henriksson et al., 2000; Langston et al., 2011; Wang and Lu, 2016).

Lytic polysaccharide monooxygenases (LPMOs), previously known as GH61 proteins, have shown enormous potential to assist hydrolysis of LCB in recent years (Basotra et al., 2019; Levasseur et al., 2013). Till date, LPMOs are classified as members of AA family 9, 10, 11, and 13, where, LPMO9s (AA9), LPMO11s (AA11), and LPMO13s (AA13) have been reported only from fungi (Levasseur et al., 2013; Loose et al., 2016). The presence of copper ion in the active site of LPMOs stimulates hydroxylation of either C1 or C4 in polysaccharide substrates forming aldonic acid or 4-keto sugars, respectively (Beeson et al., 2012; Müller et al., 2017; Walton and Davies, 2016). LPMOs require reducing equivalents to trigger their catalytic activity, and these equivalents are generated by functional electron donors like ascorbic acid or gallic acid (Quinlan et al., 2011; Vaaje-Kolstad et al., 2010) and lignin content in LCB (Walton and Davies, 2016). Several studies have shown that redox active proteins viz. CDH act as natural consort for LPMOs (Phillips et al., 2011; Rai et al., 2020; Wymelenberg et al., 2010; Yakovlev et al., 2012). Further, co-expressions of LPMOs and CDHs have also been reported in various fungi when cultured using plant biomass as a carbon source (Phillips et al., 2011; Wang and Lu, 2016).

Another set of oxidative enzymes, previously known as carbohydrate binding modules 33 (CBM33), have recently been reclassified as AA10 family proteins (Levasseur et al., 2013). CBMs are non-catalytic modules and possess carbohydrate binding activity. The lignocellulolytic enzymes are inefficient in degrading insoluble polysaccharides since they are unable to access target sites on substrates during catalysis. Therefore, cellulases/ hemicellulases are equipped with CBMs which bind cell wall polymers and increase their accessibility to lignocellulolytic enzymes (Ezeilo et al., 2017; Levasseur et al., 2013). The recent studies have shown that AA family 9 and 10 proteins act synergistically on the

crystalline portion of cellulose, and provide new ends for recognition and action of cellulases (Morgenstern et al., 2014; Vermaas et al., 2015). Therefore, these enzymes can be used for boosting the efficiency of lignocellulolytic GHs.

3.2. Microbial sources of lignocellulolytic enzymes

The lignocellulolytic (cellulase, hemicellulases, and auxiliary enzymes) enzymes producing microorganisms have been reported from diverse ecological niches including forests, compost piles, composting soils, rumens, wood processing plants, and sewage sludge (McDonald et al., 2012; Patel et al., 2019; Rai et al., 2016b). There are two most prominent lignocellulolytic systems: (i) extracellular enzymes in filamentous fungi and aerobic bacteria and (ii) an enzyme complex called as cellulosome in anaerobic bacterial and fungal strains (Arora et al., 2015b; Ezeilo et al., 2017; Mathew et al., 2008). The complexed cellulolytic systems (cellulosomes) have been reported from several anaerobic microbes, e.g., *Acetivibrio, Bacteroides, Clostidium, Ruminococcus, Nocallimastix, Piromyces,* and *Orpinomyces* (Blumer-Schuette et al., 2014; Doi and Kosugi, 2004; Fontes and Gilbert, 2010; Sadhu and Maiti, 2013). The interaction of cellulosome with cellulose is shown in Fig. 3. The non-complexed enzyme system is more common and has been widely exploited for numerous industrial applications. Several aerobic bacteria, including *Cellvibrio, Cellulomonas, Microspora, Thermobispora, Thermomonospora* sp., *Pseudomonas* sp., *Bacillus* sp., *Nocardia* sp., *Streptomyces* sp., *Erwinia chrysanthemi*, *Thermobifida fusca, Geobacillus* sp. strain WSUCF1, *Paenibacillus* sp., *Aeromonas* sp., *Aureobasidium pullulans* LB 83 have exhibited significant cellulase, hemicellulase, and auxiliary activities (Chadha et al., 2019; Islam and Roy, 2019; Pang et al., 2017; Rai, R. et al., 2019; Sadhu and Maiti, 2013). *Bacteroides luti* and *Oricola cellulosilytica* are the examples of novel cellulolytic bacteria that have been isolated from methanogenic sludge and surface of seashore, respectively (Chadha et al., 2019; Hameed et al., 2015; Shinoda et al., 2012).

Although different microbial genera are capable of producing lignocellulolytic enzymes, fungi have turned out to be the major role players (Marjamaa et al., 2013; Patel et al., 2019; Rai et al., 2016b). *Humicola grisea, H. insolens, Chaetomium thermophilum, Sporotrichum thermophila, Talaromyces emersonii, Myceliophthora thermophila, Thermoascus auranticus, Melanocarpus albomyces, Aspergillus* sp., *Trichoderma reesei, Acremonium cellulolyticus, Penicillium* sp., *Trametes versicolor,* are among the potent lignocellulolytic fungi reported over the past couple of decades (Basotra et al., 2019; Jatinder et al., 2006; Liu et al., 2013; Marjamaa et al., 2013; Phitsuwan et al., 2013; Rai et al., 2016b; Sun et al., 2019; Zambare and Christopher, 2010). Among diverse lignocellulolytic fungi, *T. reesei* and *Aspergillus* sp. have been extensively exploited as lignocellulolytic strains at commercial level (Chekushina et al., 2013; Gusakov, 2011; Rai et al., 2016b). However, recent trends have shown a paradigm shift towards other fungal strains *P. decumbens, A. cellulolyticus,* and *M. thermophila*, which exhibit better hydrolytic potential as compared to *T. reesei* at equal protein loadings (Chekushina et al., 2013; Marjamaa et al., 2013). These fungal strains are also being used at commercial level for lignocellulolytic enzymes production (Fujii et al., 2009; Gusakov, 2011; Liu et al., 2013). In addition, *P. funiculosum, P. oxalicum, Penicillium* sp. Dal5, and *P. oxalicum* 114-2 RE-10 are the potent *Penicillium* strains that have been tested and validated for efficient degradation of different pretreated biomass (Huang et al., 2015; Rai et al., 2016b; Saini et al., 2016; Yao et al., 2015).

3.3. Strain improvement strategies for hyper-producing deregulated lignocellulolytic strains

The cost of lignocellulolytic enzymes is one of the limiting factors in biorefineries; therefore, continuous efforts are being made to (i) minimize the cost of enzymes and (ii) increase overall yields of the enzymes with desired productivity. For making the concept of biorefineries economically feasible, several approaches such as random mutagenesis, site-directed mutagenesis, heterologous expression of proteins, CRISPR-Cas (Clustered Regularly

Interspaced Short Palindromic Repeats) system, genome, and metabolic engineering have been used in recent years to improve the enzymatic expression by microbial strains (Basotra et al., 2019; Fujii et al., 2018; Lim, J. et al., 2019; Misra et al., 2019; Wen et al., 2020).

Random mutagenesis is the simplest tool that has been extensively used for inducing genetic and functional modifications in the microorganisms. Several reports of cyclic mutagenesis employing physical agents such as UV radiations, chemical agents like N-methyl-N'nitro-N-nitrosoguanidine (MNNG), acriflavin, and ethanomethane sulphate (EMS), and combination of both physical and chemical mutagens have been published in past decade (Adsul et al., 2007; Chadha et al., 2005; Fang et al., 2009; Kaur et al., 2014; Lim, J. et al., 2019; Liu et al., 2013; Ottenheim et al., 2015). Reportedly, the benchmark of hypercellulolytic fungal strains has been developed through cyclic mutagenesis. The rationale behind cyclic mutagenesis is to mutate carbon catabolite repressor (*CCR*) gene in a non-specific manner that will deregulate the expression of cellulases and some hemicellulases (Amore et al., 2013; Brown et al., 2013). The alterations in carbon repressor gene *cre1,* glucosidase IIα subunit of *gls* 2α gene and β-glucosidase regulatory gene *bglR* have been reported for upregulating cellulolytic genes (Fujii et al., 2013; Geysens et al., 2005; Ilmén et al., 1996; Nitta et al., 2012). Fujii et al. (2013) reported the upregulation of cellulases and xylanase in *Acremonium cellulolyticus* by disruption of *creA* gene.

Site-directed mutagenesis is also highly accepted technique that induces specific alterations in the known DNA sequences. Error-prone PCR (EP-PCR) applied in tandem with site directed mutagenesis has been reported to increase EG activity (up to 7.93 folds) in *Bacillus amyloliquefaciens* DL-3, and enhance alkaline tolerance of EGIII in *T. reesei* (Vu and Kim, 2012; Wang et al., 2005). A study has suggested that the product of *bgl2* gene catalyses conversion of cellobiose into glucose, which in turn inhibits the expression of cellulases through feedback inhibition of *cre1* gene. Also, cellobiose acts as an inducer for

transcription factor *clrB*, which activates the expression of cellulases. Therefore, mutations in *bgl2* and *cre1* genes could significantly enhance the production of cellulases through cellobiose mediated upregulation of *clrB* gene (Yao et al., 2015).

Protoplast fusion is another technique where protoplasts isolated from two genotypically versatile somatic cells are fused into hybrid protoplast cells harbouring genetic modifications. This technique is relatively inexpensive, simple, and shows enormous potential for developing industrially competent hypercellulase strains without causing many disturbances in their physiology (Adeleye et al., 2019; Kaur et al., 2013; Savitha et al., 2010). This technique works schematically by (i) isolating protoplasts through carbohydrase (chitinase, glucanase, lysozymes, Novozyme 234) mediated digestion of cell wall, (ii) fusing isolated protoplasts (at interspecific, intraspecific, and intergeneric level) employing electrofusion or chemicals like polyethylene glycol (PEG), calcium ions, and sodium nitrate, (iii) regenerating transformed protoplasts, and (iv) screening regenerated transformants (Adeleye et al., 2019; Kaur et al., 2013; Kordowska-Wiater et al., 2012). Protoplast fusion has been used for increasing the production of cellulases in *T. reesei* (Prabavathy et al., 2006), increasing fumaric acid production from glycerol in *Rhizopus microsporus* (Kordowska-Wiater et al., 2012), developing heterokaryons of two cellulolytic strains *Aspergillus tubingensis* and *A. nidulans* (Kaur et al., 2013), and improving amylase titres of two amylolytic species of *Aspergillus* (Adeleye et al., 2019). Further, protoplast transformation technique provides another excellent platform for improving yields of cellulases and hemicellulases through RNA interference (RNAi) of *cre1/ creA* gene expression. A recent study has investigated the role of *cre1* gene in *M. thermophila* ATCC42464 where silencing of *cre1* gene through RNA interference resulted in C88 strain exhibiting up to 5.59 folds increase in cellulase activities when compared with parent strain (Yang et al., 2015). Another study reported the transformation of *Verticillium dahliae*

protoplasts with different short-interference RNAs (SiRNAs) targeting *gfp* gene. The resultant transformant SiRNA-*gfp4* exhibited significant gene silencing (up to 100%) lasting for minimum of 72 h (Rehman et al., 2016).

In recent years, genetic engineering has emerged as a powerful tool for putting together multiple traits of interest in a single organism. Therefore, this technique could be very useful in (i) increasing enzyme titer, (ii) reducing the production cost of enzymes, and (iii) developing process specific enzymes (Phillips, 2019; Singh et al., 2017). The methodology of genetic engineering involves isolation of gene of interest from the target organism, insertion of the isolated gene into a suitable vector to form recombinant DNA (rDNA), and transfer of rDNA to the expression host (Basotra et al., 2019; Rai, R. et al., 2019; Singh et al., 2017). Moreover, there are several reports where recombinant strategies have been utilized for efficient bioconversion of LCB into fermentable sugars through heterologous expression of the functional cellulase, hemicellulase, and auxiliary activity proteins (Basotra et al., 2019; Fang and Xia, 2015; Poidevin et al., 2013; Rai, R. et al., 2019). A β-glucosidase from *B. circulans* and multiple copies of bifunctional endo/exoglucanase from *Bacillus* sp. DO4 were integrated into the chromosomal DNA of *Saccharomyces cerevisiae,* and the resultant strain was reported to reduce the requirement for addition of commercial cellulases in a solid state fermentation process (Cho et al., 1999). A recombinant strain of *S. cerevisiae* was developed through expression of endoglucanase 11 and cellobiohydrolase 11 (*T. reesei*) together with β-glucosidase (*A. aculeatus*) in the form of fusion proteins attached to the cell surface (Fujita et al., 2004). The developed strain was reported to produce ~ 3 g/L ethanol from amorphous cellulose. A developed strain of *S. cerevisiae* co-expressing fungal endoglucanase EG1 (*T. reesei*) and β-glucosidase of the yeast (*Saccharomycopsis fibuligera*) has been reported as the first yeast strain capable of growing on cellulose (phosphoric acid swollen cellulose; PASC) as a sole carbohydrate source, yielding 1 g/L ethanol (Den Haan et al., 2007). The three

cellulases PaCel6A, PaCelB, and PaCel6C (*Podospora anserine*) functionally expressed in *Pichia pastoris* were reported to hydrolyse amorphous and crystalline celluloses but were found to be inactive against hydroxyethyl cellulose, mannan, galactomannan, xyloglucan, arabinoxylan, arabinan, xylan, and pectin (Poidevin et al., 2013). A study has reported the expression and production of CBH II from *T. reesei* into *P. pastoris*, and its application has also been proved in the hydrolysis of corn stover and rice straw (Fang and Xia, 2015). The cloning and expression of GH11 xylanase gene from *A. fumigatus* MKU1 has been reported where two exons of the gene were amplified separately and fused using overlap extension PCR. The fused product was then cloned in pPICZB, and expressed in *P. pastoris* under the control of AOX1 promoter (Jeya et al., 2009). Gong et al. (2013) reported cloning of *aufaeA*, a gene encoding for type-A feruloyl esterase, in *A. usamii* E001. The gene was expressed in a heterologous host *P. pastoris* GS115. One of the transformants, *P. pastoris* GSFaeA4-8 showed high expression of the recombinant *auFae A* with an enzyme activity of 10.76 U/ml. A gene encoding CDH was cloned from *Neurospora crassa* strain FGSC 2489 and successfully expressed in a heterologous host *P. pastoris* under the control of AOX1 methanol inducible promoter (Zhang et al., 2011). A novel laccase gene *pclac2* was cloned from *Phytophthora capsici* using pPIC9K expression vector and expressed into *P. pastoris* host system (Feng and Li, 2014). A thermo-alkali stable laccase was cloned from *B. licheniformis* and expressed in *P. pastoris*. The expressed protein showed remarkable stability at 70˚C with half-life ($t_{1/2}$) of 6.9 h (Lu et al., 2013). The cloning of a novel LPMO (PMO9A_MALCI) from thermophilic fungus *Malbranchea cinnamomea* and its expression in *P. pastoris* has been recently reported where the expressed AA9 protein showed hydrolysis of both cellulose and pure xylan (Basotra et al., 2019).

In case of bacteria, more reports concern introduction of cellulase and hemicellulase genes into strains of *Escherichia coli*. There is a report for the cloning of first functional gene

from *Paecilomyces thermophila* where a 681 bp xylanase gene (Pt *xynA*) was expressed in *E. coli* BL21, and the recombinant protein was purified using nickel- nitrilotriacetic acid (Ni-NTA) and Sephadex G50 columns. The characterization of the recombinant xylanase indicated that enzyme is thermostable and has a great potential in various industries (Zhang et al., 2010). A novel endo-1,4-β-D-glucanase (EG) was cloned from *T. virens* ZY-01 (Zeng et al., 2016), a cellobiohydrolase B (*cbhB*) was cloned from *A. niger* ATCC 10574 (Woon et al., 2015), soluble flavin domain of *Phanerochaete chrysosporium* CDH, and genes coding for β-xylosidase, endoxylanase, and laccase were cloned from *Geobacillus* sp. WSUCF1 through pRham N-His SUMO Kan Vector (Bhalla et al., 2014a; Bhalla et al., 2014b; Rai, R. et al., 2019) and expressed in *E. coli* and/or genetically modified *E. coli* based cells.

Epigenetics could be a handy technique when it comes to regulating gene expression. There are several factors like methylation, acetylation, and post transcriptional modifications that control expression of genes without incorporating any changes in the gene sequence (Druzhinina and Kubicek, 2017; Gibney and Nolan, 2010; Mello-de-Sousa et al., 2016). Although there is a big research gap in the area of epigenetics based strain improvement, this technique in combination with other genetic tools could be a windfall in developing industrially competent cellulase producers.

CRISPR-Cas9 is another powerful technique that has revolutionized the genetic engineering technique and is being employed successfully in several yeasts and fungi; however, its applicability is yet to be validated in case of thermophilic fungi. Liu et al. (2015) have reported CRISPR/Cas9 technology for simultaneous editing of multiple genes through co-transformation of *in vitro* synthesized gRNAs and donor DNA in *T. reesei* to enhance lignocellulose degradation. Another study conducted by Shi et al. (2016) on *S. cerevisiae* has envisioned that CRISPR technology could be a potential industrial approach for metabolic engineering.

4. Fermentation

Fermentation is a critical step for the production of industrially important fuels and chemicals where monomeric sugars released by hydrolysis of feedstock are converted into these products by the microbial action. The wild-types of microorganisms tested in the ethanol fermentation are *Calonectria brassicae, Candida (Scheffersomyces) shehatae, E. coli, S. cerevisiae, Mucorindicus, Pachysolen tannophilus, Pichia (Scheffersomyces) stipitis*, and *Zymomonas mobilis* (Sanchez and Cardona, 2008). There are many factors affecting fermentation process for bioethanol production such as temperature, pH, aeration rate, salt concentration, carbohydrate concentration, and ethanol concentration (Arora et al., 2017; Ding et al., 2020; Selim et al., 2018). There are three major modes of fermentation for ethanol production: batch, fed-batch, and continuous fermentation, and each process has its own advantages and limitations. Batch fermentation is the most traditional type of fermentation mode where high concentration of initial substrate is converted into high concentration of product, and a fresh batch is run after the end of each batch (Olsson and Hahn-Hägerdal, 1996). A modification of batch fermentation is repeated batch fermentation in which immobilized microbial cells are used instead of free ones to make the system more efficient (Jain and Chaurasia, 2014). Fed-batch method is another type of fermentation, which is a combination of batch and continuous mode, and there is intermittent addition of fresh substrates without removing products. This type of fermentation is more economical compared to batch type method due to shorter fermentation time, higher ethanol productivity, higher dissolved oxygen in media, and less toxicity of media components (Cheng et al., 2009). There is constant addition of substrate and nutrients with continuous removal of products from bioreactor in continuous type of fermentation. Continuous fermentation is the most common type of fermentation, which has been used for industrial bioethanol production due to easy process control, elimination of unproductive time required for cleaning, less

investment cost, and less labour intensive process (Kumar et al., 2015; Sanchez and Cardona, 2008).

A study is reported on the pilot-scale production of bioethanol with high yield using *Z. mobilis*, and based on techno-economic analysis, the ethanol production using this bacterium could save the cellulosic ethanol production facility by $2 million/ year (Kremer et al., 2015). Another study is reported on the pilot-scale production of bioethanol by recombinant *E. coli* FBR5 in SSF system from dilute sulphuric acid pretreated wheat straw and achieved an improved ethanol yield (0.29 g/g), and productivity (0.43 g/L/h) (Saha et al., 2015). The consolidated alcohol dehydration and oligomerization (CADO) approach, a one-step conversion process, is estimated to reduce the operating plus annual capital costs from $2.00/GJ to $1.44/GJ i.e. 28% reduction in the conversion of wet ethanol to fungible blend-stocks. This approach has enhanced the liquid hydrocarbon yield (36% of theoretical), decreased ethanol conversion cost (12-fold), and scaled up the process by 300-fold (Hannon et al., 2020). A recent study proved the enhanced bioethanol production of 20.6 g/L with volumetric productivity of 1.0 g/L/h from food waste in SSF system using the mixed culture of *F. oxysporum* F3 and *S. cerevisiae*. The supplementation of glucoamylase into mixed culture resulted in further enhancement of ethanol production and productivity by 30.3 g/L and 1.4 g/L/h, respectively, and hence, proved the feasibility of on-site production of multienzyme system and bioethanol production from food waste (Prasoulas et al., 2020). Similarly, a pilot-scale continuous tubular reactor (PCTR) technology is expected to achieve a high ethanol yield of 11.0 to 11.3 kg of ethanol per 100 Kg of untreated biomass by overcoming the challenges related to biomass recalcitrance (Pérez-Pimienta et al., 2020).

To achieve the maximum yield and productivity for bioethanol production, the selected microbial strain should have some unique features such as broad range of substrate utilization, ability to withstand under high sugar concentration, ethanol concentration, and by-

products produced during pretreatment step, and minimum by-products formation (Lugani and Sooch, 2018). However, most of the naturally occurring microbial strains employed for alcohol fermentation possess the ability to ferment hexose sugars only with very low ethanol yield and productivity. The wild pentose sugars fermenting microbial strains such as *P. stipitis, P. tannophilus,* and *C. shehatae* are sensitive to low pH, high concentration of ethanol, and inhibitors (Hahn-Hägerdal et al., 2007). Therefore, it is very difficult for wild microbial strains to fulfil the features, which are required for its selection as industrially important, and hence from last few years' focus has been given for development of genetically modified microorganisms to ensure their use for industrial applications.

4.1. Strain development for improved bioethanol production

There are various previous studies, which have been done using adaptive evolution to create mutant strains, which are resistant to high temperature, salt concentration, acetic acid concentration, freeze-thawing, pentose sugars, and various stress inducers (Abreu-Cavalheiro and Monteiro, 2013; Choe et al., 2019; Sharma, N.K. et al., 2016; Sharma et al., 2017; Stanley et al., 2010; Wati et al., 1996). The powerful tools used to develop industrial important fermenting microbial strains to meet the demand of alcoholic fuel are genetic engineering, recombinant DNA technology, metabolic engineering, cell surface engineering, protein engineering, protoplast fusion, and CRISPR-Cas9. The other techniques used for manipulating the microbial genes to improve the efficiency of saccharification and fermentation are adaptation, selection, mutation, and protoplast fusion. The tools used for selection of genetically modified ethanol resistant strains are deletion mutant library screening and transposon-mediated mutant collection (Teixeira et al., 2009; Zheng et al., 2011). The ethanol resistant strains are produced by global transcription machinery engineering (gTME), which is a powerful tool for selection of mutant library (Yang et al., 2011). Many *in-silico* tools like dynamic flux balance model and dynamic simulations are

used for analysis of bioethanol production by genetically modified microorganisms in co-culture fermentation (Parambil and Sarkar, 2015). The main focus of developing genetically modified microbial strains is to accelerate the rate of reaction, shifting the existing metabolic pathway towards production of useful products, enhancing substrate specificity, altering enzyme activity for producing novel structures (Doğan et al., 2014). Many previous studies have already been reported on the production of enhanced bioethanol using recombinant microbial strains (Abreu-Cavalheiro and Monteiro, 2013; Doğan et al., 2014; Ge et al., 2014; Ko et al., 2018; Kricka et al., 2014; Sar et al., 2017). The recombinant bacterial strains named *E. coli* and *Klebsiella oxytoca* showed the ability to utilize wide range of substrates with enhanced bioethanol production (Dien et al., 2003). Previously, it has been observed that *S. cerevisiae* mutant with disrupted *ura7* or *gal6* showed increased resistance to different kind of stressors including ethanol. The mutant yeast strain also showed enhanced glucose consumption at low temperature compared to wild strains (Yazawa et al., 2007). Both ethanol tolerance and fermentation capacity of sake yeast strains were enhanced by over expression of *msn2* (Watanabe et al., 2009). The enhanced effective and rapid ethanol production (with 90% of maximum theoretical yield) was achieved with *Geobacillus thermoglucosidasius* by up-regulating the expression of pyruvate dehydrogenase, and disruption of pyruvate formate lyase and lactate dehydrogenase genes (Cripps et al., 2009). The overexpression of sugar transporter (Hxt) in *Fusarium oxysporum* resulted in enhanced glucose and xylose transport capacity with 39% increase in ethanol yield (Ali et al., 2013).

Cell surface engineering is an innovative tool in molecular breeding for displaying functional proteins on the surface of microorganism used in consolidated bioprocessing (CBP) system. Cell display system is very useful for ethanol production from starch in CBP because various amylases are displayed on the yeast cell surface, which can utilize starch as the sole carbon source for ethanol production (Sakuragi et al., 2011). The recombinant

thermophilic strain of *Kluyveromyces marxianus* has been developed using cell surface engineering, and the recombinant strain displayed both β-glucosidase and endoglucanase on cell surface. The recombinant strain was used for bioethanol production in CBP system using β-glucan as substrate, and the improved bioethanol production of 0.47 g/g of carbohydrate consumed was observed at the end of fermentation (Hasunuma and Kondo, 2012). *E. coli* is considered as one of the important industrial bacteria, and is commonly used for most of the recombinant studies. Xylose metabolic pathway was introduced into *Z. mobilis* from *E. coli* for producing recombinant strain having GRAS (Generally Recognized as Safe) status. The recombinant strain showed minimum nutrients requirement, and could tolerate high temperature and low pH (McEwen and Atsumi, 2012). Similarly, constitute promoter substitution and xylose metabolic integration was done in *S. cerevisiae* for producing engineered strain EBY101-X5CC, and the engineered yeast strain had the ability for co-fermentation of cellulose and either sucrose or xylose. The recombinant strain produced 4.3 g/L ethanol from 10 g/L carboxymethyl cellulose (CMC) in CBP system (Li et al., 2017). The engineering of both feedstock as well as microorganism resulted in enhanced bioethanol production. This strategy also provides the feasibility of ethanol production at commercial scale using lignocellulosic waste materials (Ko et al., 2018). In a recent study, the safe and stable expression of cellulase gene (*sestc*) and glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) promoter was achieved using CRISPR -Cas9 approach in *S. cerevisiae* chromosome using gRNA expression vector from *Agaricus biporus*. The recombinant yeast strain showed increased expression of endo-1,4-β-glucanase and exo-1,4-β-glucanase, and 37.7-fold improved ethanol production compared to its native strain (Yang et al., 2018). In a recent study, industrially engineered *S. cerevisiae* MF01-PHO4 was produced by protoplast formation and *pho4* gene replacement, and mutant strain was observed to be stable up to 30 generations. An enhanced ethanol yield of 114.71 g/L was achieved with the genetically

engineered strain, accounting for 5.30% increase in ethanol yield and 12.5% decrease in fermentation time (Wu et al., 2020). There is no clear evidence on side effects of genetically modified microorganisms on environment; thus, there is still a need to take preventive measures to ensure environmental safety. Federal government renewable fuel standards (FGRFS) should be adapted before introducing genetically modified microorganisms on large scale bioethanol production. A practically engineered core modelling tools hold promises for biosafety of genetically modified microorganisms in bioethanol production to sustain biotechnology advancement.

4.2. Strain development for co-fermentation of glucose and xylose

The conversion of LCB to ethanol is associated with the challenges such as co-utilization of pentose and hexose sugars, and presence of fermentative inhibitory compounds such as phenolic derivatives, acetic acid, and furfurans. However, few strains such as *S. shehatae*, *S. stipitis,* and *K. marxianus* have the ability to assimilate the pentose sugars but the production of ethanol is not up to the industrial standards, and also co-fermentation of pentose and hexose sugars possess a major obstacle in efficient conversion of LCB to ethanol (Arora et al., 2015c; Kim et al., 2012; Moysés et al., 2016).

Several organisms are capable of converting sugars into ethanol, but *S. cerevisiae* is majorly used at the industrial scale for ethanol production due to its versatile characteristics such as high ethanol tolerance, ability to withstand low pH, ability to ferment under anaerobic condition, tolerance to high osmotic pressure, and less prone to bacteriophage infections (Robak and Balcerek, 2018). The yeast *S. cerevisiae* exhibits weak expression of pentose pathway gene, and have poor/ no xylose uptake ability. Over the last few years, considerable development in genetic engineering has changed the metabolic engineering paradigm. Specialized tool boxes are available for pathway manipulation of several strains by

overexpression and knock out of genes targeting metabolic pathways, molecular transport capability, cellular tolerance, and catabolite sensing (Selim et al., 2018).

4.3. Xylose metabolism

The pentose sugar xylose is metabolized by the microorganisms during xylose metabolism using two different pathways. In filamentous yeasts, oxidoreductase pathway having two-step reaction is involved. In the initial step, the xylose is reduced to xylitol through NAD(P)H and/or NADH dependent xylose reductase (XR) (EC 1.1.1.30) encoded by *xyl1, xyl1p*, and in the second step, the xylitol is oxidized to xylulose by NAD^+ dependent xylitol dehydrogenase (XDH) (EC 1.1.1.9) encoded by *xyl2, xyl2p*. In case of bacteria, xylose is directly converted into 5-xylulose using xylose isomerase (XI) (EC 5.3.1.5) encoded by *xylA* without any co-factor usage. The 5-xylulose is phosphorylated to xylulose-5-phosphate with xylulokinase (XK) (EC 2.7.1.17), which is an intermediated for phosphoketolase pathway and non-oxidative pentose pathway. The pentose pathway can be further classified into two distinct pathways, namely non-oxidative and oxidative pentose phosphate pathways. Most of the yeasts use non-oxidative pathway to metabolize xylulose-5-phosphate as a precursor for nucleic acid and amino acids and also convert to three, four, five, six and seven atom carbon sources, which serves as intermediate to glycolysis. While the oxidative pathway is used as defensive mechanism against oxidative stress and to generate NADPH, which is a major precursor for biomass formation, and also driving element of XR (Karhumaa et al., 2007; Kwak and Jin, 2017).

4.3.1. Utilization of xylose by engineering oxidoreductase pathway

Even though *S. cerevisiae* encodes for putative pentose pathway genes, the expression level of these genes is weak, and is unable to assimilate xylose as sole carbon source. Hence, there is a requirement of heterologous complementation and significant metabolic engineering. Among the pentose utilizing strains, *S. stipitis* is the most studied organism as its

xylose pathway of converting pentose sugars to ethanol is well curated (Harner et al., 2015). Most of the heterologous expression genes related to pentose pathway were used from *S. stipitis* as compared to other eukaryotic organisms. Kötter et al. (1990) isolated *xyl1* and *xyl2* genes encoding XR and XDH from *S. stipitis* genomic DNA and cloned in *S. cerevisiae* for the first time, and showed oxidative utilization of xylose. Later, ample work has been carried on expressing *xyl1* and *xyl2* genes from *S. stipitis* in *S. cerevisiae* (Ho et al., 1998; Kim et al., 2013; Toivari et al., 2001), and reported higher assimilation of xylose and ethanol production as compared to engineered strain overexpressed with XI gene (Karhumaa et al., 2007; Li et al., 2016). The XR-XDH is oxidoreductases based enzyme, and require balance for complete assimilation of xylose. Under anaerobic condition, the NADH cannot be re-oxidized to NAD^+ using oxygen as terminal electron acceptor; hence, there is excess accumulation of NADPH and reduced NAD^+ availability. Further the XR in *P. stipitis* has higher affinity for NADPH (K_m= 3.2 µmol/L) as compared to NADH (K_m=40 µmol/L) and XDH is completely relied on NAD^+, which causes severe disparity in the redox balance, and leads to excess xylitol production and reduced ethanol production (Jeffries and Jin, 2004).

To overcome the xylitol accumulation due to the redox imbalance, several strategies were employed including addition of external electron acceptor such as acetoin, acetaldehyde, furfural, and 5-hydroxymethylfurfural (HMF). Addition of acetoin and furfural showed reduced xylitol accumulation and increased ethanol production from 0.62 mol ethanol/mol xylose to 1.35 mol ethanol/mol xylose by decreasing the flux by oxidative pentose pathway as the reduction of acetoin and furfural required NADH (Wahlbom and Hahn–Hägerdal, 2002). Almeida et al. (2009) reported that overexpression of gene encoding furaldehyde reductase as co-factor used in HMF reduction significantly influenced the ethanol production. They reported that the NADH-dependent reductase exhibited carbon conservation by reducing glycerol formation and enhanced NAD^+ availability for XDH,

which ease the xylose uptake and reduced xylitol accumulation. In *S. cerevisiae,* ammonium assimilation involves glutamate dehydrogenase. Basically, glutamate dehydrogenase catalyzes the synthesis of glutamate from ammonium and 2-ketoglutarate. In *S. cerevisiae,* two glutamate dehydrogenases namely NADPH-dependent glutamate dehydrogenase and NADH dependent glutamate dehydrogenase are responsible for ammonium assimilation. Co-factor imbalance in the recombinant *S. cerevisiae* can be reduced by modifying ammonium assimilation by deletion of *gdh1* encoding NADPH-dependent glutamate dehydrogenase (EC 1.4.1.4) and the overexpression of *gdh2* encoding NADH-dependent glutamate dehydrogenase (EC 1.4.1.2), which improved the ethanol yield from 0.43 to 0.51 cmol/cmol while by reducing the xylitol accumulation by 44%. The increase in ethanol production and reduced xylitol production is due to increase in the NAD^+ availability for XDH, which directed the xylose towards product and biomass formation (Roca et al., 2003). The re-oxidation of NADH can be achieved by channelizing the carbon flux through recombinant phosphoketolase pathway. Overexpression of phosphotransacetylase and acetaldehyde dehydrogenase in combination with the native phosphoketolase in xylose fermenting *S. cerevisiae* strain TMB3001c showed reduced glycerol and xylitol accumulation, while increasing the ethanol yield by 25% (Sonderegger et al., 2004).

The accumulation of xylitol was reduced when glucose is used as a co-substrate; however, this costs in reduced xylose assimilation due to the competition among the sugar transporters (Hallborn et al., 1994). In yeasts consisting of XDH to XR ratio of 1.66 showed higher ethanol productivity with low xylitol formation rate as compared to XR. A recombinant *S. cerevisiae* strain with XR:XDH ratio of 0.06 showed no xylitol and acetic acid formation, and depicted a good ethanol yield as compared with the strain with higher XR:XDH ratio (Walfridsson et al., 1997). Multicopy integration of *xyl2* gene encoding XDH in the recombinant *S. cerevisiae* strain elevated the xylulose accumulation and reduced the

xylitol formation reflecting that the activity of XK inhibits the assimilation and utilization of xylose on such cells (Jeffries and Jin, 2004). Further inefficient XK activity will tend to accumulate excess xylulose, and reduced intracellular levels of ATP and the ATP/ADP ratio with the subsequent overexpression of XR, XDH, and XK substantially enhance the production of ethanol in *S. cerevisiae* (Richard et al., 2000). The engineered *S. cerevisiae* strain consisting of XR isozyme for wild type, and mutant showed ethanol yield of 0.47 g/g emphasizing the role of XR in increasing the ethanol yield (Jo et al., 2017).

4.3.2. Utilization of xylose by engineering isomerase pathway

The XI pathway is evident in most of the bacterial species as compared to yeast and in contrast to oxidoreductase pathway, this enzyme does not require co-factor and convert xylose directly to xylulose. Most of the heterologous expression of XI in *S. cerevisiae* strain showed lower functionality; this might be due to sub-optimal internal pH, absence of specific metal ion, post-translational modification and protein misfolding. Heterologous expression of XI from *Piromyces* sp. E2 (*pirXI*), an anaerobic fungus, increased the flux of xylose towards ethanol production; however, misfolding of the protein was evident which restrained the enzyme activity (Lee et al., 2017). Co-expression of cytoplasmic chaperonin complex Gro EL-Gro ES complex from *E. coli* in the recombinant *S. cerevisiae* cloned with XI from the bacterium *Propionibacterium acidipropionici* displayed proper folding of XI and efficiently converted xylose to ethanol with the yield of 0.44 g ethanol/g xylose (Temer et al., 2017).

Walfridsson et al. (1996) expressed *xylA* gene encoding XI from *Thermus thermophiles*, which showed high specific activity (1.0 U/ mg of protein) at 80°C but had poor performance (with specific activity of 0.04 U/ mg of protein) at 30°C, and accumulated xylitol and acetate as by-product. On the similar lines, Lönn et al. (2003) performed a study by overexpressing multicopy *xylA* gene from *Thermus thermophiles* and found xylitol formation by activity of non-specific endogenous aldose reductase (GRE3) which reduced the activity of non-

oxidative pentose pathway and XK. The deletion of *gre3* and over-expression of an extra copy of XK in the recombinant strain improved the ethanol productivity and reduced the xylitol production. The bottleneck related to xylitol accumulation and increasing the xylose flux was addressed by over-expressing enzymes such as xylulokinase (EC 2.7.1.17), ribulose 5-phosphate isomerase (EC 5.3.1.6), ribulose 5-phosphate epimerase (EC 5.3.1.1), transketolase (EC 2.2.1.1), and transaldolase (EC 2.2.1.2) with the deletion of GRE3. The engineered strain showed simultaneous uptake of glucose and xylose (Kuyper et al., 2005). The affinity of XI from *Ruminococcus flavefaciens* towards xylose can be improved by adapting modifications to the 5'-end of the gene, site-directed mutagenesis, and codon optimization. The modified enzyme showed 4.8 fold higher activity as compared to the native enzyme with the K_m= 66.7 mM and specific activity of 1.41 µmol/min/mg. The recombinant *S. cerevisiae* harbouring the modified enzyme along with cellobiose phosphorylase, cellobiose transporters, the endogenous genes *gal2* (encoding transporter gene) and *xk* and disruption of the native *pho13* (encoding p-nitrophenylphosphatase) and *gre3* genes resulted in four-fold higher xylose consumption even in the presence of lignocellulosic inhibitors and showed higher ethanol concentration (Aeling et al., 2012). Likewise, the xylose consumption can be increased by overexpressing the heterologous sugar transporter (*PsSUT1*) and *XK* in the engineered strain containing *xylA* from the fungus *Orpinomyces,* showed ethanol yield of 0.48 g/g and low xylitol yield of 0.04 g/g when grown in complex medium supplemented with 0.01M borate (Madhavan, et al., 2009). Brat et al. (2009) compared the performance of XI isolated from anaerobic bacterium *Clostridium phytofermentans* and *Piromyces sp.,* and reported low inhibition of xylitol in the strain cloned with *C. phytofermentans* XI with the ethanol yield of 0.43 g/g and xylitol production of 0.18 g/g. Cloning and expression of XI gene (*xylA*) of *Burkholderia cenocepacia* in *S. cerevisiae* showed better co-consumption of glucose and xylose under anaerobic condition and also resulted in higher ethanol yield of

0.45 g/g without xylitol accumulation (Peng et al., 2015). Ota et al. (2013) showed that the cell surface display of *xylA* from *C. cellulovorans* with the over-expression of *XK* resulted in 0.5 g/g ethanol yield under anaerobic condition.

To overcome the challenges related to redox imbalance, some studies have been focused on altering NADH/NADPH ratio for the efficient performance of the XR and XDH. Since NADPH co-factor is majorly generated through the oxidative pentose phosphate pathway. Deletion of *zwf1* (glucose-6-phosphate dehydrogenase, EC 1.1.1.49) and *gnd1* (6-phosphogluconate dehydrogenase, EC 1.1.1.44) genes reduced the xylitol production with low XR/XDH ratio. However, the mutant having *Δzwf1* and *Δgnd1* showed reduced xylitol yield and growth rate due to significant drop in NADPH levels.

To subsidize the negative effect caused by deletion of *zwf1,* over-expression of *gpd 1* encoding $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase (*Kluyveromyces lactis* GPD 1, EC 1.2.1.13) resulted in 52% alleviation in ethanol yield and 48% lower xylitol accumulation (Verho et al., 2003). The overexpression of water forming NADH oxidase (EC 1.6.99.3) gene *noxE* from *Lactococcus lactis* in recombinant *S. cerevisiae* lead to significant decrease in glycerol and xylitol production, and hence increased final ethanol production during xylose metabolism. The ethanol yields of 0.294 g/g and 0.211 g/g, respectively, were observed with recombinant and control strains of *S. cerevisiae*, which clearly revealed the effect of cofactor imbalance on the production of by-products i.e. ethanol and xylitol (Zhang et al., 2012). The improvement of xylose uptake and ethanol production by over-expression of oxidoreductase and xylose isomerase pathways is shown in Table 4.

4.3.3. Engineering of transporters for xylose uptake

Yeast shows an efficient transport system for the endogenous metabolism of hexose sugars but limited exogenous xylose metabolism and has a low affinity as it is dependent on the hexose transport system. Considerable efforts have been made to engineer the xylose

transporters to improve the simultaneous uptake of hexose and pentose sugars (Sharma, N.K. et al., 2018). The strength of the xylose transporter can be improved by targeting and engineering the existing sugar transporters or searching novel heterologous xylose affinity/glucose repressor-based transporter (Kwak and Jin, 2017). *S. cerevisiae* has 18 hexose transporters among which Hxt 1-17 and Gal2 are responsible for glucose permeation across the cell membrane, while Hxt 1-7 acts as glucose facilitator. Several hexose transporters such as Hxt1, Hxt2, Hxt4, Hxt5, Hxt7, and Gal2 facilitate the xylose uptake in *S. cerevisiae*; however, these transporters have low affinity towards xylose in the presence of glucose and show inefficiency in transportation at lower xylose concentration (Hamacher et al., 2002; Saloheimo et al., 2007). Among the hexose transporters, Hxt7 and Gal2 have shown higher affinity for xylose, but in the presence of glucose these transporters had repressed and reduced xylose uptake rate.

The two transporters in the yeast are described namely Gxs1 (glucose/xylose symporter) and Gxf1 (glucose/xylose facilitator). To enhance the uptake of xylose in *S. cerevisiae* Leandro and co-workers expressed *C. intermedia* PYCC 4715 transport proteins (Gxs1 and Gxf1) in *S. cerevisiae*. The recombinant strain exhibited a higher growth rate in xylose containing medium with K_m=0.2mM, but in the presence of glucose in the medium, the affinity towards xylose was significantly reduced. These results concluded that the activity of transporter proteins Gxs1 and Gxf1 is directly proportional to the glucose concentration (Leandro et al., 2006). Young et al. (2011) expressed *C. intermedia* Gxs1 and Gxf1 along with *S. stipitis* Xut1 and Xut2 in hexose null mutant which barely showed any improvement on xylose containing medium. When Gxf1 was expressed in *S. cerevisiae,* the recombinant strain showed higher xylose uptake at lower concentration of xylose, but it was unchanged even at higher concentration of xylose in aerobic condition. The strain also exhibited higher ethanol production and xylose uptake under anaerobic condition (Runquist

et al., 2009). Young et al. (2011) created a mutant of transporter protein *C. intermedia* Gxs1 and *S. stipitis* Xut1 through directed evolution method and expressed these mutant genes in hexose null *S. cerevisiae* mutant. The recombinant strain showed substantial growth and uptake of xylose in the glucose/xylose medium. Further improvement in V_{max} and K_m was observed by point mutating amino acid, Phe40 in Gxs1 and Glu538 in Xut1. Similarly, the single nucleotide polymorphism was created by point mutating Phe79Ser in HXT7, which showed a co-utilization of glucose and xylose sugars with higher xylose uptake ability with V_{max}=186.4 nmol/ml/min as compared to wild type with V_{max}=101.6 nmol/ml/min (Apel et al., 2016). Based on the sequence similarity of Gxs1 with other xylose transporters, a conserved motif sequence G-G/F-XXX-G has been identified and successive mutation in the amino acids Phe38, Ile39 and Met40 showed two-fold improvement in the xylose uptake rate (Young et al., 2014).

The presence of three sugar transporters Sut1, Sut2, and Sut3 in *P. stipitis* showed higher affinity towards glucose than xylose; however, Sut1 has higher V_{max} for xylose as compared to other transporters. Over-expression of *P. stipitis* Sut1 in recombinant *S. cerevisiae* harbouring XR-XDH-XK genes showed higher uptake of xylose in glucose/xylose fermentation with an ethanol yield of 0.44 g/g sugar (Katahira et al., 2008). Goncalves et al. (2014) over-expressed Hxt1, Hxt2, Hxt5, and Hxt7 permeases in the hexoses null mutant strain (*hxt1Δ-hxt7Δ* and *gal2Δ*) consisting *xyl1, xyl2* and *xk* genes. The results revealed that Hxt7 has higher xylose consumption ability compared to other transporters; however, the substrate affinity was 200 fold higher for glucose as compared to xylose in the medium containing glucose/xylose mixture making xylose second choice even in the presence of low concentration of glucose. While Hxt1 showed higher sugar uptake and ethanol productivity in co-fermentation of glucose and xylose but severely repressed the xylose uptake in the presence of glucose showing diauxic growth profile. To overcome the barrier related to

transporter repression, Farwick and co-authors (Farwick et al., 2014) conducted homology modelling for xylose transporters to transport D-xylose without any inhibition by D-glucose. This study showed that glucose-insensitive xylose transporters could be obtained by mutations in Gal2 and Hxt7 transporters, and hence contributed to understand sugar-transport mechanisms. Single point mutation in N376-F region of Gal2 and N370-S region of Hxt7 displayed higher affinity to xylose and lost their ability to transport hexose sugars. Nijland et al. (2017) adapted an evolutionary engineering strategy to develop a chimeric HXT36 by fusion of functional hexose transporter Hxt3-Hxt6. An amino acid substitution at N367A of Hxt36 has enabled the co-consumption of glucose and xylose. The genome sequence analysis showed that co-repressor such as CYC8 and SSN6 are responsible for phenotypic characteristics of non-evolved strain. Inactivation of CYC8 showed higher activity of Hxt, which in turn increased the xylose transport and less sensitive to D-glucose repression (Nijland et al., 2017). Wei et al. (2018) found 11 transcriptional factors in glycolysis and pentose pathway of yeast that varied with the concentration of xylose and glucose/xylose in the medium. Knockout of THI2 promoted ribosome synthesis, enhanced the xylose uptake rate and ethanol production by 26.8 and 32.4%, respectively. Also, the over-expression of cell cycle related transcriptional factor Nrm1 further improved the xylose utilization rate by 30% and ethanol production by 76.6% in glucose and xylose containing medium. The overview of metabolic engineering in yeast for simultaneous uptake of glucose and xylose is depicted in Fig. 4.

4.3.4. *Elimination of by-products for efficient production of ethanol*

In microbial fermentation the production of by-products are inevitable, which in turn divert the carbon flux from the main product and thereby reducing the desired product titers (Arora et al., 2019). In order to overcome this bottleneck, appropriate rewiring of metabolic pathway is indispensable. In *S. cerevisiae*, glycerol is one of the major by-products, which accounts

for 2-3% of sugar bioconversion. Even though glycerol is one of the platform chemical separation during ethanol fermentation is not economically viable (Prior and Hohmann, 1997). In *S. cerevisiae,* the glycerol formation is a two-step process, In the initial step, the NADH- dependent glycerol-3-phosphate dehydrogenases (GPD) catalyze the conversion of dihydroxyacetone phosphate to glycerol-3-phosphate followed by dephosphorylation of glycerol-3-phosphate to glycerol (Gancedo et al., 1968; Påhlman et al., 2001). Glycerol is usually accumulated in the cell during osmotic stress condition and acts as osmolytes (Luyten, 1995). Jain et al. (2011) eliminated the *gdp1* (osmotically induced) and *gdp2* (anaerobically induced) but the growth of the strain was hindered under anaerobic conditions due to the excess accumulation of NADH. The redox imbalance was mitigated by introduction of oxido-reductase gene, which convert NADH to NAD+ by production of sorbitol and propane-1,2-diol and ethanol yield was maintained at 0.48 g/g glucose. In the similar lines, Papapetridis et al. (2017), deleted *gdp2* and aldehyde dehydrogenase (*ALD6*) genes and replaced it with native *gdp* of an archaeal NADP+-preferring enzyme in an acetate reducing *S. cerevisiae* strain. The mutant strain was able to grow under anaerobic conditions with high osmolarity with the consumption of acetic acid without producing glycerol. Acetic acid is another product usually observed in *S. cerevisiae* fermentation. Acetic acid is also one of the major inhibitors present in lignocellulosic hydrolysate. Wei et al., 2013 proposed co-utilization of xylose and acetic acid for the production of ethanol by combining the NADH – producing xylose utilization pathway and NADH consuming acetate reduction pathway. For this, they deleted *gdp1* and *gdp2* in order to reduce the glycerol formation and introduced XR-XDH from *P. stipitis* and *adhE* and *mphF* (proteins that are part of a bifunctional aldolase–dehydrogenase complex involved in 4-hydroxy-2-ketovalerate catabolism) from *E. coli*. The *adhE* and *mphF* gene aided in reduction of acetate to ethanol by generating 2 NAD+ molecules. In xylose assimilation pathway, 1 mole of NADH is generated by the oxidation of

xylitol to D-xylulose. The co-factor is exchanged between these two pathways showing improved ethanol production.

5. Integration of steps

The most extensively used method of ethanol production using LCB is separate hydrolysis and fermentation (SHF), which involves two consecutive steps of enzymatic hydrolysis and fermentation in separate reactors. In this process, there is optimization of each process separately to achieve better enzymatic hydrolysis and microbial fermentation. However, some of the major limitations associated with this method are high production cost, less product yield, and high chances of contamination.

The fermentation of hexoses and pentoses are performed in different reactors during SHF, which further increase the processing time (Chandel et al., 2007; Offei et al., 2018; Tandon and Sharma, 2019). Based on the limitations associated with SHF, various integration processes such as simultaneous saccharification and fermentation/ co-fermentation (SSF/SSCF) and consolidated bioprocessing (CBP) approaches have been adapted for commercial production of bioethanol (Fig. 5) (Arora et al., 2015a). CBP encompasses two strategies i.e. engineering of wild microbial strains to improve product related (titre and yield) properties, and expression of heterologous cellulase system for cellulose utilization by high product yielding non-cellulolytic microbes (Lynd et al., 2005).

5.1. Simultaneous Saccharification and Fermentation/ Co-fermentation (SSF/SSCF)

In SSF and SSCF processes, both enzymatic hydrolysis and fermentation processes are carried out simultaneously in the same reactor to maintain low concentration of glucose as the accumulation of glucose inhibits the enzymatic activity. The fermentation of hexoses is performed by hexose fermenting microorganisms in SSF whereas, both pentoses and hexoses are fermented in the same reactor in SSCF. In this process, the saccharification of cellulose and fermentation/ co-fermentation of pentose and hexose sugars do not occur simultaneously,

but in a sequential manner (Zhao et al., 2011). These integrated approaches are less susceptible to contamination due to immediate conversion of sugars into ethanol in the same reaction vessel, which also leads to higher ethanol yields due to avoidance of feedback inhibition to enzyme. Moreover, these approaches have easy process design, short reaction time, easy to operate with reduced process cost. However, there are some limitations associated with these processes. One of the major challenges associated with this integrated approach is optimization of reaction conditions to make the system more efficient because separate optimal conditions are required for enzymatic hydrolysis and microbial fermentation. SSF and SSCF processes are operated normally at 30-35^{o}C to accommodate microbial growth and ethanol fermentation (Azhar et al., 2017; Canilha et al., 2012; Nikolić et al., 2016). Moreover, the fermentation media used for bioethanol production is very viscous in nature due to presence of lignin content of LCB, and it is very difficult to separate lignin from cellulosic part before fermentation which results in difficulty in heat and mass transfer, and homogenous mixing of culture and media components. Hence, the energy consumption is high for distillation of fermentation broth and treatment of distillate (Zhao et al., 2011). The promising microorganisms for bioethanol fermentation in SSF system are *S. cerevisiae* and *Z. mobilis* (Nigam and Singh, 1995). The other yeast strains, which have been reported for bioethanol production in SSF system are *K. marxianus, K. fragilis, P. pastoris,* and *Hansenula polymorpha* (Mejía-Barajas et al., 2016). It has been found that microwave-assisted liquefaction (power of 80 W for 5 min duration) of cornmeal (cornmeal to water ratio of 1:3) increased the bioethanol production by 13.4% using *S. cerevisiae* var. *ellipsoideus* in SSF process (Nikolić et al., 2016). In another study, SSF system was used for bioethanol production from recycled paper sludge using *P. stipitis* CBS 5773. Celluclast®1.5 L supplemented with Novozym®188 was used for enzymatic hydrolysis, which resulted in 100% saccharification. The ethanol concentration of 19.6 g/L was achieved after 179 h of

fermentation (Marques et al., 2008). Similarly, bioethanol production was reported using pretreated municipal solid waste via SSF using *S. cerevisiae* in fed-batch mode with 25% (w/w) substrate loading and achieved an ethanol concentration of 30 g/L (Ballesteros et al., 2010). Recently, a novel pretreatment method namely cosolvent enhanced lignocellulosic fractionation (CELF) has been developed by Wyman and Dale (2015) to improve the ethanol yield by recovering all the sugars from hemicellulose and remove 90% of lignin from biomass. Previously, thermotolerant yeast strain *S. cerevisiae* KNU5377 was used for ethanol production from pretreated waste newspaper (250 g/L, solid loading) in SSF system, and the ethanol production of 8.4% (v/v) was obtained at 50ºC after 72 h in 5 L fermenter (Park et al., 2010). In another study, SSF was conducted using *S. cerevisiae* under shaking conditions (60 rpm) using 1% (v/v) inoculum under semi-anaerobic conditions for ethanol production from dates juice, and 88% substrate was converted into bioethanol at the end of fermentation with product yield of 0.51 g/g sugar (Taouda et al., 2017). In a recent study, bioethanol production of 82.1 g/L was reported using sulphite pretreated momentary pine slurry (25%, w/w) in SSF system. Pre-hydrolysis was done at 50°C for 24 h and 200 rpm followed by fermentation at 28°C or 35°C using 5 g/L dry inoculum of *S. cerevisiae* (Dong et al., 2018). Many previous studies have been reported on bioethanol production using SSCF fermentation (Erdei et al., 2013; Liu and Chen, 2016; Qin et al., 2018; Sharma, S. et al., 2018). SSCF was used to improve ethanol production and productivity of 48.63 g/L and 2.03 g/L/h, respectively, from food waste using *S. coreanus* and *P. stipitis* (Jeong et al., 2012).

5.2. Consolidated Bioprocessing (CBP)

The concept of consolidated bioprocessing (CBP) strategy was evolved from direct microbial conversion, but wild microbial strains are not available for commercial bioethanol production using this approach (Zhao et al., 2011). In CBP, all the steps of bioethanol production, i.e., enzymes production, cellulose hydrolysis, and fermentation, are conducted in

a single vessel, and single microbial community is used for both production of cellulases and fermentation, which makes the process cost effective. Lynd and his team have made great contribution on developing CBP systems for industrial ethanol production with high yield and titre from native and recombinant microbial strains (Holwerda et al., 2014; Hon et al., 2018; Izquierdo et al., 2014; Laser et al., 2002; Lynd et al., 2005; Olson et al., 2012; Tian et al., 2017; Van Zyl et al., 2007). In CBP, rational designs are used for production of engineered strains of bacteria and yeast with high ethanol titre (Jin et al., 2011). Filamentous fungi, *Fusarium oxysporum,* possesses the potential of bioethanol production from lignocelluloses in CBP system (Ali et al., 2016). In the past few years, thermophilic anaerobic cellulolytic bacteria such as *T. ethanolicus, C. thermohydrosulfuricum, T. mathranii, Thermoanaerobium brockii*, and *C. thermosaccharolyticum* have been explored for bioethanol production using CBP due to their ability for direct conversion of cheaper biomass feedstocks into bioethanol at extreme temperature. However, these extremophiles are sensitive to ethanol concentration, which is a major hurdle for their use (Lynd et al., 2005; Vazirzadeh and Robati, 2013). The economic commercial production of bioethanol (66 million gallons at a breakeven price of $1.31 per gallons) from pure sugarcane bagasse feed has been reported by (Raftery and Karim, 2017). In another study, pine needle biomass was pretreated using ionic liquid followed by fermentation using *S. cerevisiae* and *P. stipitis* in CBP system. The ethanol yield of 0.148 g/g has been obtained after 72 h, and the fermentation efficiency of system was found at 41.39% (Vaid et al., 2018). Recently, recombinant *S. cerevisiae* ER T12 and M2n T1 strains (containing integrated temA and temG Opt gene cassettes) strain simultaneously expressing α-amylase and glucoamylase produced 89.35 g/L and 98.13 g/L ethanol from starchy biomass with carbon conversion of 87% and 94%, respectively, in a single step CBP system at 30°C after 192 h (Cripwell et al., 2019). Max-min driving force (MDF) algorithm was used for thermodynamic analysis of ethanol production from cellobiose in *Clostridium*

thermocellum, and the improved ethanol production has been found by maintaining the negative free energy change for all metabolic steps involved in the pathway (Dash et al., 2019). Another study conducted by Beri et al. (2020) has proved the consumption of 85% recalcitrant glucuronoarabinoxylan (GAX) from isolated *Herbinix* spp. strain LL1355, and six enzymes are found to be involved in the hydrolysis of GAX linkage. The successful expression of up to four genes in *Thermoanaerobacterium thermosaccharolyticum* increased the GAX consumption and ethanol yield of 78% and 28%, respectively, from corn fiber. Recently, cell-free extract reaction (CFER) system has been developed from *Clostridium thermocellum* to identify potential metabolic engineering systems for enhancing ethanol titer (Cui et al., 2020). Although CBP method is much improved for ethanol production compared to other existing methods due to less production cost of enzymes, yet there is some gap on commercial use of CBP systems. Future studies has been recommended for understanding the metabolic pathways of miroorganisms, synergistic action between the microbes and their enzymes for simultaneous pretreatment, hydrolysis and fermentation, and developing recombinant strains and biodesign strategies for enhanced ethanol production with improved yields.

6. Future Prospects

Lignocellulosic ethanol is an environmentally friendly, renewable oxygenated fuel with minimum emission of GHGs during combustion. In recent years, many new technologies have been developed for cost-effective bioethanol production at industrial scale. Nevertheless, there are still some challenges such as development of efficient pretreatment technologies, stable performance of genetically modified organisms, and integration of optimal components for cellulosic ethanol production at commercial level. The cost of bioethanol production can be decreased either by reducing the cost of feedstock or enzymes used for hydrolysis step or by developing the integrated process configurations. The choice of

pretreatment method relies on the type of biomass being selected for clean 2G sugars production at competitive prices. Thermo-mechanical extrusion method is considered one of the efficient methods of pretreatment which can be used in combination with other technologies such as particle-size reduction and green solvent pretreatment for efficient ethanol production. Pretreatment cost and chemical waste generation can also be reduced by altering lignin structure of LCB and expressing novel microbial enzymes in plants, which results in decreased molecular weight of lignin without compromising the biomass yield.

Enzyme hydrolysis is more convenient than acid hydrolysis; however, this process is expensive due to high enzyme cost. Hence, on-site production of enzymes could be an effective strategy to reduce the production cost of bioethanol. The innovative technologies like protein engineering and computational protein design can be used for generation of cost-effective and industrially important novel biocatalysts. The ethanol production efficiency can also be improved by using genetically modified microbes, which possess the ability to ferment pentose and hexose sugars simultaneously in the presence of fermentation inhibitors by eliminating the detoxification step. Metabolic engineering, cell surface engineering, and synthetic biology are other promising approaches, which are being used for the synthesis of engineered host fermentation system to improve the production of bioethanol. CRISPR- Cas9 is a simple but powerful gene-editing tool for safe and stable gene expression, which can be used for synthesis of engineered microbial strains. The enzymatic conversion can be enhanced by tailoring of reactor design, which is also used to overcome rheological and bioconversion inhibition.

CBP is more efficient for economic bioethanol production because all the steps are performed in a single reactor by a single microorganism capable of producing both hydrolytic enzymes and fermentation. Development of biorefineries is also need of the hour for economical utilization of lignocelluloses. Focusing on a single product or bioethanol may not

be economically viable option. A biorefinery may be design in such a fashion to valorize its each and every component of lignocelluloses into biofuels and biochemicals for sustainable development of circular bioeconomy (Chandel et al., 2020). Therefore, the focus should be given to develop such kind of feasible systems to reduce production cost and improve production yields. Although several technologies have already been investigated to increase production of ethanol, still most of them are under laboratory scale, and hence, a comprehensive economic and process analysis is required to develop strategy for industrial bioethanol production to solve the problem of energy crisis in a sustainable manner.

7. Concluding Remarks

Lignocellulosic or second generation (2G) ethanol is being considered as one of the long term sustainable alternative to the depleting crude oil reserves. However, there are several technical and economic challenges associated with success of bioethanol refineries. Low cost pretreatment to overcome biomass inherent recalcitrance in an eco-friendly manner is the first major hindrance that needs to be addressed. High cost associated with the commercially available cellulase/ hemicellulase enzymes is another bottleneck that could be addressed by formulating indigenous tailor made enzyme cocktails that are highly efficient against a wide range of agro-residues even at low protein loadings. The future research should have target on designing integrated approaches for simultaneous pretreatment, saccharification of biomass, and fermentation of the released sugars. The third major challenge in 2G ethanol processing is the limited uptake of xylose by fermenting yeasts in the presence of glucose. Therefore, the future research may be focused on the development of robust engineered yeast having the transporters for simultaneous uptake of glucose and xylose with equal assimilation rates. Addressing these challenges may help scientific community to provide solutions for escalating global energy demand together with mitigating climate issues in a sustainable manner.

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Figure captions

Fig. 1. Different pretreatment approaches, and their pros and cons.

Fig. 2. Lignocellulolytic enzymes in action on plant cell wall

Fig. 3. Cellulosome in action for saccharification of cellulose (Modified from Arora et al. (2015b))

Fig. 4. Metabolic Pathway engineering for xylose assimilation in *Saccharomyces cerevisiae*. Dark Green represents the gene deletion and Blue represents the heterologous overexpression of genes. XR: Xylose Reductase, XDH: Xylitol dehydrogenase, XKS: xylulose kinase, XI: Xylose isomerase, ZWF1 glucose-6-phsophate dehydrogenase, GND1 6-phosphogluconate dehydrogenase, RPE1: Ribulose phosphate 3 epimerase, noxE; NADH dependent oxidase, DHAP: Dihydroxyacetone phosphate, G3P: Glyceraldehyde 3 Phosphate, PDC: pyruvate decarboxylase, ADH: Aldehyde dehydrogenase, and Transporter: Hxt7, Gal2, Gxs, Gxf.

Fig. 5. Integration approaches for commercial production of bioethanol. Where SHF: Separate hydrolysis and fermentation; SSCF: Simultaneous saccharification and fermentation; SSCF: Simultaneous saccharification and co-fermentation; CBP: Consolidated bioprocessing.

Table 1: Recent publications (2015-2020) on bioethanol production from utilization of lignocellulosic biomass

S.No.	Review Title	Description of Review	Reference
1.	Recent advances in pretreatment technologies for efficient hydrolysis of lignocellulosic biomass	Composition of LCB, effective parameters limiting the hydrolysis of lignocelluloses, pretreatment technologies for LCB to obtain fermentable sugars for ethanol production	(Akhtar et al., 2016)
2.	Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: Concepts and recent developments	Bioethanol as an ecofriendly biofuel including different feedstocks, their structural organization, different pretreatment methods, fermentation of sugars into ethanol, product recovery, optimization of saccharification and fermentation bioprocess, economic considerations for cellulosic ethanol production	(Saini et al., 2015)
3.	Lignocellulosic biomass: A sustainable platform for the production of bio-based chemicals and polymers	Structure and sources of LCB, valuable chemicals from LCB, existing, planned and under construction facilities to produce bioethanol from LCB	(Isikgor and Becer, 2015)
4.	Lignocelluloses: An economical and ecological resource for bio-ethanol production- A review	Composition of LCB, potential sources of agro-based wastes, microorganisms and their lignolytic enzymes, overview on LCB conversion into bio-ethanol, pretreatment methods, hydrolysis of pretreated biomass, fermentation, methods used to improve fungal enzyme production, activity and/or stability	(Allen et al., 2016)
5.	Consolidated briefing biochemical ethanol production from lignocellulosic biomass	Current status of global bioethanol production, sources and composition of LCB, processing routes to bioethanol including pretreatment, hydrolysis and fermentation, recent issues in bioethanol production including gap between biotech research and commercialization and bioethanol based economy	(Spyridon and Willem Euverink, 2016)
6.	Utilization of agricultural waste for bioethanol production- A review	Classification of agricultural wastes, conversion of agricultural waste to ethanol, hydrolysis of cellulose in the LCB, fermentation of sugar to ethanol, pretreatment technologies for agricultural waste with advantages and disadvantages of each method	(Nwosu-Obieogu et al., 2016)
7.	Recent progresses in bioethanol production from lignocellulosic materials: A review	Different feedstocks used for bioethanol production, lignocellulosic and algal feedstocks for bioethanol production, bioethanol processing from LM, different pretreatment and hydrolysis methods for LCB, fermentation including integrated approaches (SHF, SSF, SSCF, SSFF, CBP), ethanol recovery, and LCF biorefinery	(Haq et al., 2016)
8.	Bioethanol production from lignocellulosic waste- A review	Different methods of pretreatment for agricultural wastes including wheat straw, rice straw, corn straw and bagasse, enzymatic hydrolysis, and fermentation	(Mohanty and Abdullahi, 2016)
9.	A prospective of bioethanol production from biomass ass alternative fuel for spark ignition engine	Types and feedstocks for different generations of biofuels, process for conversion of biomass to bioethanol, fuel properties of bioethanol, engine performance and emissions using bioethanol and its blends	(Sebayang et al., 2016)

10.	A review on current technological advancement of lignocellulosic bioethanol production	Production of bioethanol from LCB including pretreatment, hydrolysis and fermentation, integrated approaches (SHF, SSF, CBP) used in fermentation with special emphasis to SSF, yeast genetic engineering, ethanol production from different thermotolerant yeasts with comparison of ethanol yield from different strains	(Sharma, V. et al., 2016)
11.	A review on second and third generation bioethanol production	Different generations of biofuels with their feedstocks, processes for production of second and third generation bioethanol, biocatalysis of ethanol, cogeneration of energy from sugarcane, life cycle assessment of ethanol and gasoline	(Teixeira et al., 2016)
12.	Fuel ethanol production from lignocellulosic biomass: An overview on feedstocks and technological approaches	Sources and composition of LCB, conversion of LCB into ethanol using pretreatment, detoxification, hydrolysis, fermentation and product recovery, strategies used to overcome inhibitors problems during ethanol production, integration processes used for bioethanol production, role of microorganisms in pretreatment, detoxification, hydrolysis and fermentation	(Zabed et al., 2016)
13.	Current status and strategies for second generation biofuel production using microbial systems	LCB pretreatment methods, second generation biofuel production from biomass using microbes, different approaches to enhance biofuel production, and current production status of bioethanol	(Bhatia et al., 2017)
14.	Insight into progress in pretreatment of lignocellulosic biomass	An overview on biochemical route including different processes for bioethanol production, challenges, advantages and recent developments in different pretreatment processes, quantitative comparisons of leading pretreatment technologies, process integration to establish commercial systems,	(Bhutto et al., 2017)
15.	A review on the pretreatment of lignocellulose for high-value chemicals	Structure of lignocellulose, pretreatment methods with their action mechanisms for extraction of sugars (pentoses and hexoses) from LCF, existing problems for pretreatment of lignocelluloses	(Chen et al., 2017)
16.	Recent updates on different methods of pretreatment of lignocellulosic feedstocks: A review	Composition of common LCF, different pretreatment processes, their advantages and limitations, production of value added products i.e. biofuels and chemicals from pretreated LCB	(Kumar and Sharma, 2017)
17.	Recent status on enzymatic saccharification of lignocellulosic biomass for bioethanol production	An overview of different processes used in conversion of LCB into ethanol, enzymes used for lignocellulosic hydrolysis, production of cellulose and hemicellulose by microorganisms, various factors affecting enzymatic hydrolysis	(Madadi et al., 2017)
18.	Review on pretreatment methods and ethanol production from cellulosic water hyacinth	Composition of LCB, cell wall composition of water hyacinth (WH), pretreatment methods with advantages and disadvantages, sugar production from WH, fermentation and ethanol production with recent studies on ethanol production from WH	(Rezania et al., 2017)
19.	Harnessing the potential of bio-ethanol production from lignocellulosic biomass in Nigeria- A review	Potential LCB feedstocks in Nigeria, bioethanol production from sugarcane bagasse, corn cobs, mango peels, sorghum straw, and rice husks, cellulosic biomass capacity for bioethanol production in Nigeria, pathways to bioethanol production, challenges of LCB conversion to bioethanol, and commercialization of biomass to bioethanol process	(Awoyale and Lokhat, 2019)

20.	Recent trends in the pretreatment of lignocellulosic biomass for value-added products	Structure of LCB and its components, different pretreatment methods for extraction of reducing sugars for production of biofuels including bioethanol, biogas, aldehydes, phenols and organic acids	(Baruah et al., 2018)
21.	Engineering ligninolytic consortium for bioconversion of lignocelluloses to ethanol and chemicals	Engineering of ligninolytic armory, enzymatic hydrolysis and fermentation, strategies for improvement of lignocellulosic ethanol production, lignocellulose-derived platform chemicals	(Bilal et al., 2018)
22.	Effects of organosolv pretreatment conditions for lignocellulosic biomass in biorefinery applications: A review	Organosolv pretreatment method with advantages and disadvantages, optimal pretreatment conditions for effective delignification and enzymatic conversion, organosolv based biorefineries	(Borand and Karaosmanoğlu, 2018)
23.	Second generation bioethanol production: On the use of pulp and paper industry wastes as feedstock	LCB composition, production of second generation bioethanol using pretreatment, hydrolysis/ saccharification, fermentation, recovery and dehydration, bioethanol production from kraft pulp, spent suLCFite liquor and paper and pulp sludge, and conversion of paper and pulp mills into biorefineries	(Branco et al., 2019)
24.	Bioethanol production from renewable raw materials and its separation and purification: A review	Biorefinery and bioethanol production, raw materials and their pretreatment for bioethanol production, bioethanol production from raw materials containing sugar, starch and LCB, bioethanol separation and purification	(Bušić et al., 2018)
25.	Emerging role of nanobiocatalysts in hydrolysis of lignocellulosic biomass leading to sustainable bioethanol production	Conventional methods for hydrolysis of LCB, role of nanobiocatalysts in hydrolysis, different nanomaterials used in nanobiocatalysis, synthesis of nanocellulose, toxicity concerns of nanoparticles,	(Rai, M. et al., 2019)
26.	Current methodologies and advances in bio-ethanol production	Bioethanol production from LCB, bioethanol production using molecular tools and genetically modified organisms	(Rastogi and Shrivastava, 2018)
27.	Review of second generation bioethanol production from residual biomass	Types of bioethanol generations, composition of LCF, conversion of LCB into ethanol using different types of pretreatment methods, enzymatic hydrolysis and fermentation, SHF, SSF, SSCF, CBP , pentose fermentation and xylose metabolism, recombinant fermentative microbes, distillation and dehydration for ethanol recovery, and new improvements in ethanol production from LCB	(Robak and Balcerek, 2018)
28.	Conversion of lignocellulosic biomass to bioethanol: An overview with a focus on pretreatment	Composition of LCB, overall bioconversion process of LCB into ethanol, comparison of conventional and developing pretreatment methods (1996 to 2018) for different LM with advantages and limitations of each method, SSCF process for bioethanol production	(Singh and Satapathy, 2018)
29.	Assessment of different pretreatment technologies for efficient bioconversion of lignocellulose to ethanol	Composition of LCB, whole process of bioethanol production including pretreatment, hydrolysis, fermentation and distillation, integrated approaches for bioethanol production (SHF, SSF, SSCF, CBP), advantages and disadvantages of various physical, physico-chemical and biological pretreatment methods, hurdles in pretreatment of LCB	(Singh and Satapathy, 2018)
30.	Bioethanol production from lignocellulosic biomass by environment-friendly pretreatment methods: A review	Composition of various LCB, ethanol recovery from different feedstocks, pretreatment techniques of LCB	(Tayyab et al., 2018)

31.	Lignocellulosic biomass for bioethanol: An overview on pretreatment, hydrolysis and fermentation processes	Sources and components of LCB, production steps of bioethanol including pretreatment and hydrolysis methods, fermentation process, integrated processes in bioethanol production,	(Abo et al., 2019)
32.	A review on commercial-scale high-value products that can be produced alongside cellulosic ethanol	Production of cellulosic ethanol in a step-wise manner, production of different inhibitors during enzymatic hydrolysis and fermentation and their impact, opportunities to produce bio-based chemicals alongside cellulosic ethanol, technology readiness level (TRL) of chemicals which have reached to commercial scale	(Rosales-Calderon and Arantes, 2019)
33.	Cellulose solvent-based pretreatment for enhanced second-generation biofuel production: A review	Different generations and types of biofuels, structure of LCB, biomass recalcitrance and pretreatment, hydrolysis of pretreated biomass, obstacles in the enzymatic hydrolysis of lignocelluloses and the role of pretreatment, comparison of IL, CPA and NMMO pretreatment methods	(Satari et al., 2019)
34.	Bioethanol from microalgal biomass: A promising approach in biorefinery	Overview on different steps for production of first, second, third and fourth generation biofuels, details on production of production of fourth generation biofuels including cultivation and accumulation of carbohydrates, harvesting, recycling of water and nutrients after cultivation, saccharification and fermentation, distillation, concentration, transportation and use, comparison of bioethanol productivities from plants and microalgae	(Silva and Bertucco, 2019)
35.	An overview on bioethanol production from lignocellulosic feedstocks	Physico-chemical properties of bioethanol, steps involved in bioethanol production, feedstocks for bioethanol production, fermentation process and mechanism, types of fermentation in bioethanol production (SHF, SSF, BF, FBF, CF, SoSF, NSSF, SSCF, SSFF, CBP), advantages of CBP	(Toor et al., 2020)
36.	Microbial delignification and hydrolysis of lignocellulosic biomass to enhance biofuel production: An overview and future prospect	Chemical composition of different LCB, comparison of different pretreatment methods, factors affecting biological pretreatments, delignification using bacteria, fungi and their enzymes with action mechanisms, hydrolysis of polysaccharides from LCB, fermentation, lignocellulose fuel economy	(Tsegaye et al., 2019)
37.	Bioethanol production techniques from lignocellulosic biomass as alternative fuel: A review	Structure and composition of LCB, process for conversion of LCB into ethanol, processes used in different pretreatment methods with their advantages and disadvantages, integrated fermentation approaches (SHF, SSF, SSCF) for ethanol production with advantages and disadvantages of each process, effect of different LCB, pretreatment and hydrolysis methods and conditions, and fermentation conditions on ethanol yield, use of bioethanol as an alternative fuel	(Abdu Yusuf and L Inambao, 2019)
38.	Contemporary pretreatment strategies for bioethanol production from corncobs: A comprehensive review	Composition of corncobs, contemporary pretreatment strategies, formation of inhibitory by-products and minimizing their effects, chemical hydrolysis	(Arumugam et al., 2020)
39.	Pretreatment methods for lignocellulosic biofuels production: Current advances, challenges and future prospects	Different LCF and their compositions, conventional and advanced pretreatment technologies with advantages and limitations of each method	(Cheah et al., 2020)

40.	Lignocellulosic bioethanol production: Prospects of emerging membrane technologies to improve the process- A critical review	Existing processes of lignocellulosic bioethanol production, conventional fermentation technology to produce bioethanol, conventional separation process and limitations of conventional processes, emerging membrane processes for lignocellulosic bioethanol production, lignocellulosic substrates for membrane technology based bioethanol production, membrane based advanced enzymatic saccharification and fermentative bioethanol production, advanced membrane separation processes for recovery of bioethanol, advanced SSFF strategy for lignocellulosic bioethanol production, status of lignocellulosic bioethanol production at international and national level, economic aspects of lignocellulosic bioethanol production	(Dey et al., 2020)
41.	Different pretreatment technologies of lignocellulosic biomass for bioethanol production: An overview	LCB structure, steps for conversion of LCB into ethanol, pretreatment methods with merits and demerits of each method, efficiency of different pretreatment methods for conversion of LM into ethanol with treatment and fermentation conditions, ethanol yield from different raw materials using different combinations of pretreatments	(Rezania et al., 2020)
42.	Current state-of-the art in ethanol production from lignocellulosic feedstocks	Structure of LCB, pretreatment strategies for removal of lignin and xylan, detoxification of LCH by various methods, enzymatic hydrolysis, fermentation, distillation and dehydration,	(Robak and Balcerek, 2020)
43.	Recent advances in bioethanol production from lignocelluloses: A comprehensive focus on enzyme engineering and designer biocatalysts	Green pretreatment approaches including use of ILs and DES used for pretreatment of LCB, enzymatic saccharification of LCB, microbial sources of lignocellulolytic enzymes, strain improvement strategies for hyper-production of lignocellulolytic strains, fermentation and integrated approaches for ethanol production, strain development for co-fermentation of xylose and glucose, pathway and genes involved in xylose metabolism, utilization of xylose by engineering of xylose isomerase pathway, engineering of transporters for improved xylose uptake, pilot-scale systems available for ethanol production	This study

Table 2: Literature review on the effects of ionic liquid pretreatment on lignocellulosic biomass conversion

S. No.	Biomass	Ionic liquid	Pretreatment conditions	Effect on saccharification	References
1	Dirty cotton residue (DCR) and cotton filter powder (CFP)	1-ethyl-3-methylimidazolium acetate	140 °C, 2 h, solid-to liquid ratio (1:9 w/v)	For DCR: Glucan to Glucose yield:78%, xylose yield: 94.9 %, delignification 45.5% For CFP: Glucan to glucose yield:75.8%, Xylan to xylose yield: 95.7 %, delignification 16%	(Fockink et al., 2020)
2	Rice straw	1-H-3-methylmorpholinium chloride	120°C, 5h, solid loading (5% w/w), 50% water	Hydrolysis yield increased from 33.2% to 70.1%	(Mohammadi et al., 2019)
3	Eucalyptus	Tetrabutylammonium hydroxide	60°C, 30 min, solid loading (10%, w/v)	Reducing sugar yield of 426.6 mg/g	(Wang et al., 2018)
4	Bamboo biomass	1-butyl 3-methylimidazolium chloride	120°C, 6 h, solid loading (20%, w/v)	Reducing sugar yield of 80%	(Kassaye et al., 2017)
5	Oil palm frond biomass	1-ethyl-3-methylimidazolium diethyl phosphate	100°C, 4 h, solid-to liquid ratio (1:10, w/v)	Lignin reduction by 55%; no enzymatic reaction performed	(Financie et al., 2016)
6	Switchgrass	1-butyl-3-methylimidazolium methanesulfonate	110°C, 4 h, solid loading (5%, w/v)	23% reduction in cellulose crystallinity; 12 g/l glucose recovery on employing 2% pretreated switch grass	(Xia et al., 2014)
7	Rice straw	Cholinium lysine	90°C, 5 h, biomass loading (5%, w/w)	Glucose yield:84.0%, Xylose yield:42.1%	(Hou et al., 2012)

8	Switchgrass	1-ethyl-3-methylimidazolium acetate	160°C, 3 h, biomass loading (3%, w/w)	Reduced saccharification time from 72 h to 12 h	(Li et al., 2010)

Table 3: DES literature review on the effects of DES pretreatment on lignocellulosic biomass conversion.

Biomass	Deep eutectic solvent	Pretreatment conditions	Effect on saccharification	References
Oil palm empty fruit bunch	Choline chloride-lactic acid	120°C, 3 h, solid to liquid ratio (1:10, w/v)	Reducing sugars yield: 20.7%	(Thi and Lee, 2019)
Eucalyptus saw dust	Choline chloride-lactic acid	110°C, 6 h, solid loading (10%, w/v)	Hydrolysis yield: 94.3%, delignification 80%	(Shen et al., 2019)
Rice straw, rice husk and wheat straw	Choline chloride-Oxalic acid-n butanol	120°C, 1 h, solid loading (15%, w/v)	Delignification:50%	(Kandanelli et al., 2018)
Switchgrass	Choline chloride- p-coumaric acid	160°C, 3 h	Glucose yield: 85.7%, Xylose yield: 28.8%, Delignification:60.8%	(Kim et al., 2018)
Oil palm fronds	Choline chloride-Urea-$CuCl_2$	120°C, 30 min, solid loading (10%, w/v)	Xylose yield: 14.76 g/L	(Loow et al., 2018)
Lettuce residue	Choline chloride-glycerol	150°C, 16 h, solid:liquid ratio (1:16, w/v)	Glucose yield: 94.9%, Xylose yield: 75.0%	(Procentese et al., 2017)

Oil Palm Empty Fruit Bunch	Choline chloride-Urea	110°C, 1 h, solid to liquid ratio (1:5, w/v)	Glucose yield: 66.33 mg/ml	(Nor et al., 2015)
Corn stover	Choline chloride-Formic acid	130°C, 2h, solid loading (5% w/v)	Glucose yield: 99%, Lignin removal: 23.8%	(Xu et al., 2016)
Corncob	Choline chloride-Imidazole	115°C, 15 h, solid to liquid ratio (1:16, w/v)	Glucose yield: 94%, Xylose yield: 84%	(Procentese et al., 2015)
Rice husk	Ethylene glycol - choline chloride	160°C, 4 h, solid loading (4%, w/v)	Reducing sugar yield: 0.74 mg/ml	(Nagoor Gunny et al., 2014)
Rice straw	Potassium carbonate-glycerol	140°C, 100 min, solid to liquid ratio (1:10, w/v)	Enhanced delignification	(Lim, W.-L. et al., 2019)

Table 4: Over-expression of heterologous pentose phosphate pathway genes for the improvement of xylose consumption and ethanol yield.

Strain	Gene over-expressed	Xylose consumption rate (g/g/h)	Ethanol yield (g/g)	Ethanol yield of wild type strain (g/g)	References
S. cerevisiae	XR, XDH, XK	-	0.11	0.0	(Toivari et al., 2001)
S. cerevisiae	XR, XDH, XK	0.28	0.24	0.0	(Kim et al., 2013)
S. cerevisiae	XR, XDH, XK	0.76	0.4	-	(Cadete et al., 2016)
S. cerevisiae	XR, XDH, XK	0.129	0.378	-	(Li et al., 2016)
S. cerevisiae	XI, XK	-	0.41	0.010	(Kuyper et al., 2005)
S. cerevisiae	XI, XK	1.87	0.41	-	(Zhou et al., 2012)
S. cerevisiae	XI, XK	0.98	0.45	0.025	(Lee et al., 2014)

*XR: xylose reductase; XDH: xylitol dehydrogenase; XI: xylose isomerase

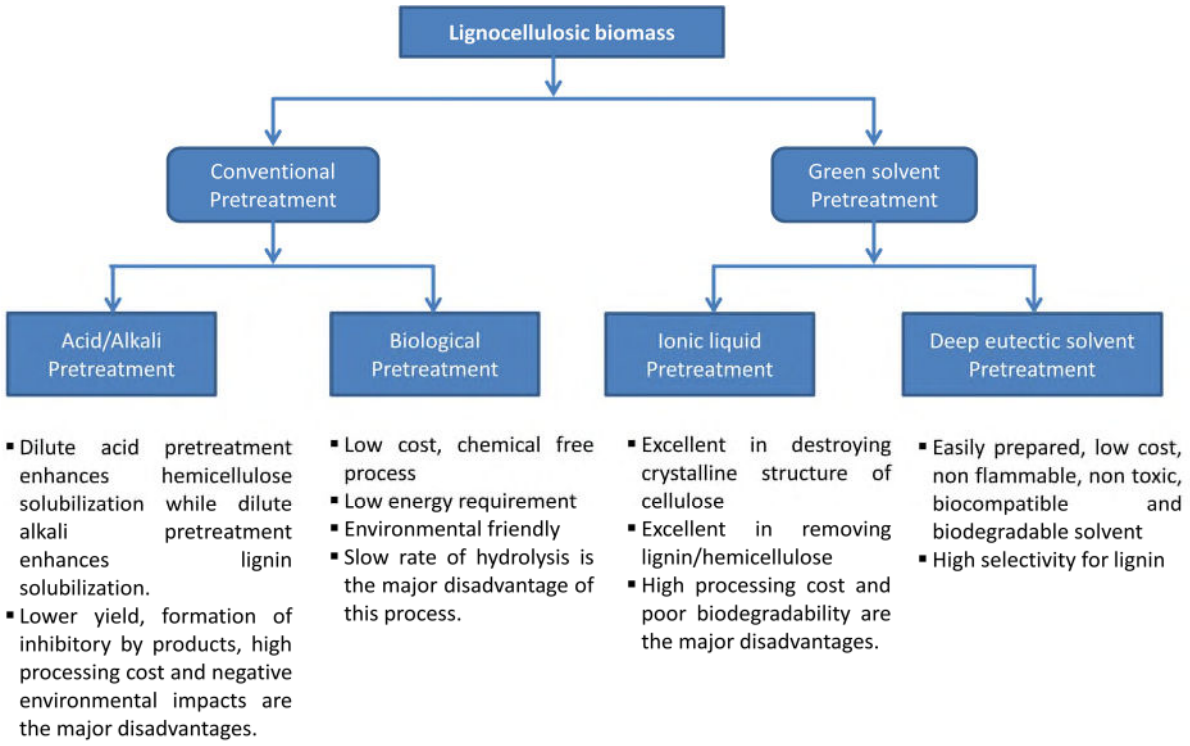

Lignocellulosic biomass
Conventional Pretreatment
Green solvent Pretreatment
Acid/Alkali Pretreatment
Biological Pretreatment
Ionic liquid Pretreatment
Deep eutectic solvent Pretreatment
▪ Dilute acid pretreatment enhances hemicellulose solubilization while dilute alkali pretreatment enhances lignin solubilization.
▪ Lower yield, formation of inhibitory by products, high processing cost and negative environmental impacts are the major disadvantages.
▪ Low cost, chemical free process
▪ Low energy requirement
▪ Environmental friendly
▪ Slow rate of hydrolysis is the major disadvantage of this process.
▪ Excellent in destroying crystalline structure of cellulose
▪ Excellent in removing lignin/hemicellulose
▪ High processing cost and poor biodegradability are the major disadvantages.
▪ Easily prepared, low cost, non flammable, non toxic, biocompatible and biodegradable solvent
▪ High selectivity for lignin

Fig. 1.

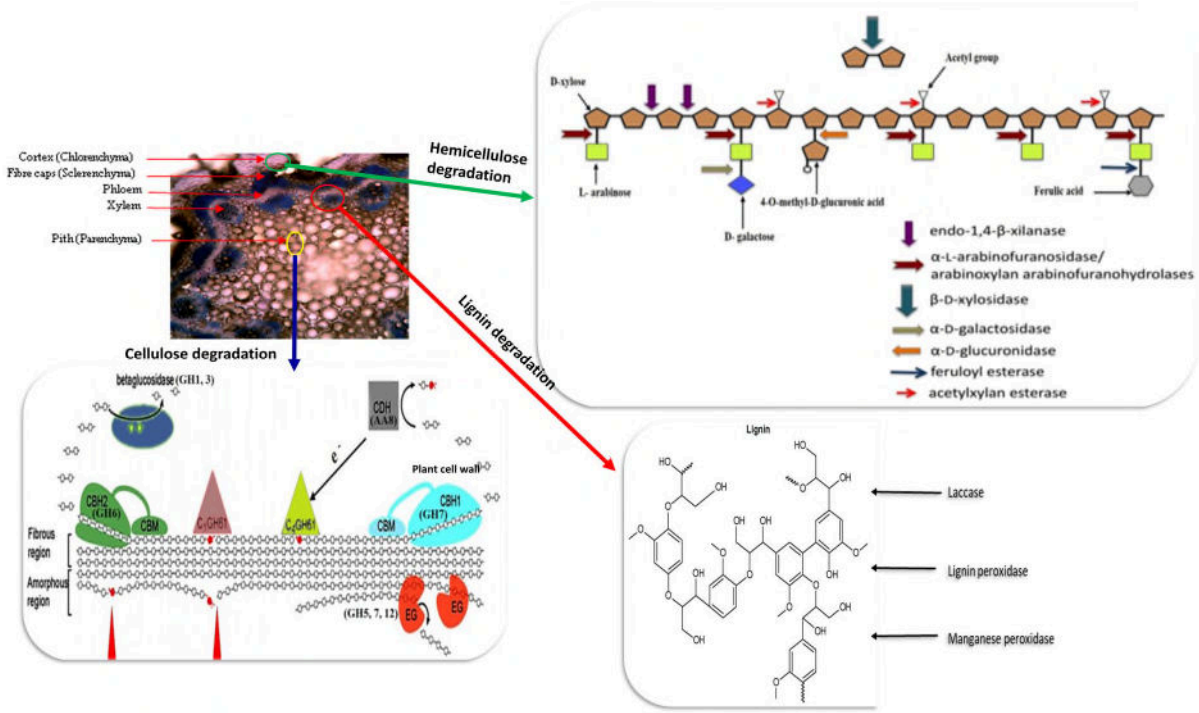

Cortex (Chlorenchyma)
Fibre caps (Sclerenchyma)
Phloem
Xylem
Pith (Parenchyma)
Hemicellulose degradation
Lignin degradation
Cellulose degradation
betaglucosidase (GH1, 3)
CDH (AA8)
Plant cell wall
CBH2 (GH6)
CBM
CBH1 (GH7)
CSM
Fibrous region
Amorphous region
(GH5, 7, 12)
EG
D-xylose
Acetyl group
L- arabinose
4-O-methyl-D-glucuronic acid
D- galactose
Ferulic acid
endo-1,4-β-xilanase
α-L-arabinofuranosidase/ arabinoxylan arabinofuranohydrolases
β-D-xylosidase
α-D-galactosidase
α-D-glucuronidase
feruloyl esterase
acetylxylan esterase
Lignin
Laccase
Lignin peroxidase
Manganese peroxidase

Fig. 2.

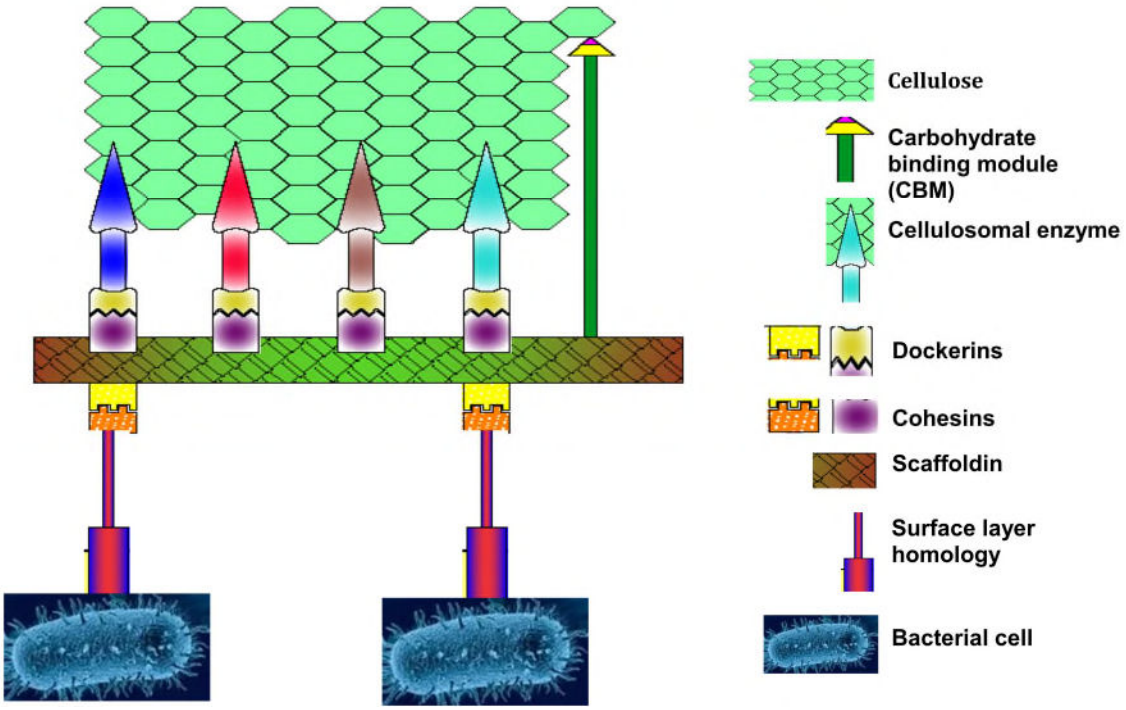

Cellulose
Carbohydrate binding module (CBM)
Cellulosomal enzyme
Dockerins
Cohesins
Scaffoldin
Surface layer homology
Bacterial cell

Fig. 3.

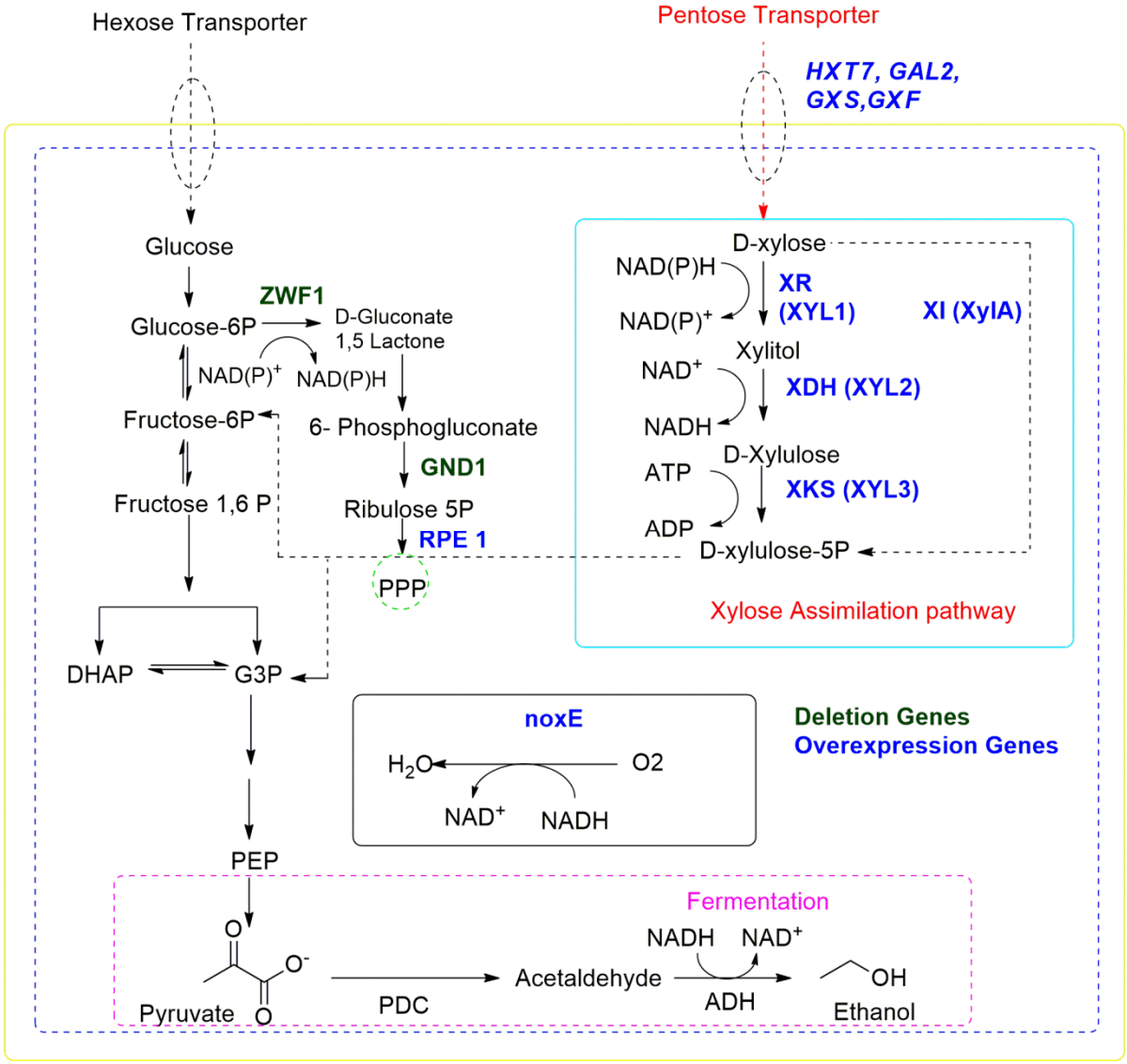

Hexose Transporter
Pentose Transporter
HXT7, GAL2,
GXS,GXF
Glucose
ZWF1
Glucose-6P
D-Gluconate
1,5 Lactone
NAD(P)+
NAD(P)H
Fructose-6P
6- Phosphogluconate
GND1
Fructose 1,6 P
Ribulose 5P
RPE 1
PPP
DHAP
G3P
D-xylose
NAD(P)H
NAD(P)+
XR
(XYL1)
XI (XylA)
Xylitol
NAD+
NADH
XDH (XYL2)
D-Xylulose
ATP
ADP
XKS (XYL3)
D-xylulose-5P
Xylose Assimilation pathway
noxE
H2O
O2
NAD+
NADH
Deletion Genes
Overexpression Genes
PEP
Fermentation
Pyruvate
PDC
Acetaldehyde
NADH
NAD+
ADH
OH
Ethanol

Fig. 4.

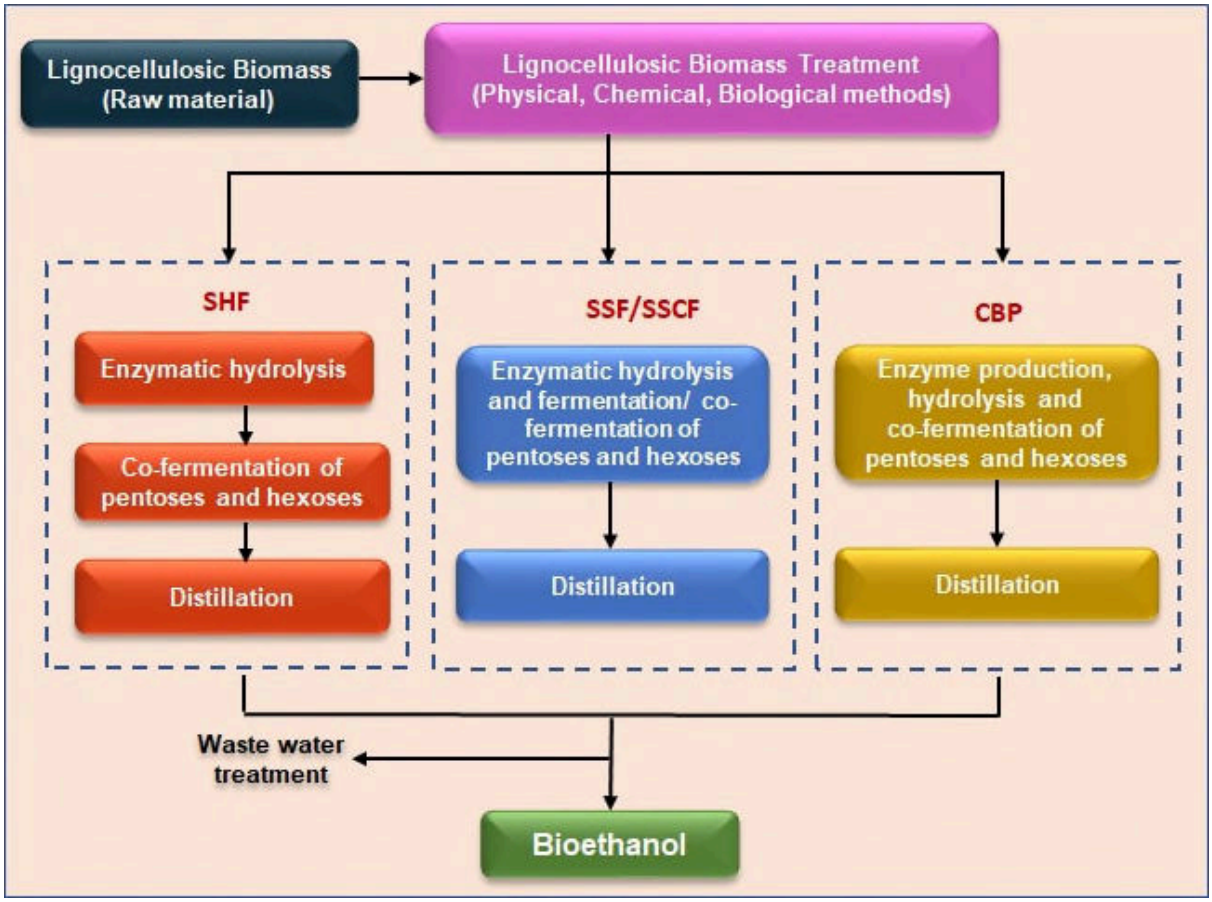

Lignocellulosic Biomass
(Raw material)
Lignocellulosic Biomass Treatment
(Physical, Chemical, Biological methods)
SHF
Enzymatic hydrolysis
Co-fermentation of
pentoses and hexoses
Distillation
SSF/SSCF
Enzymatic hydrolysis
and fermentation/ co-
fermentation of
pentoses and hexoses
Distillation
CBP
Enzyme production,
hydrolysis and
co-fermentation of
pentoses and hexoses
Distillation
Waste water
treatment
Bioethanol

Fig. 5.



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Recent advances in bioethanol production from lignocelluloses: a comprehensive review with a focus on enzyme engineering and designer biocatalysts

Lugani, Yogita; Rai, Rohit; Prabhu, Ashish A.; Maan, Poonam; Hans, Meenu ...

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