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Review Paper

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

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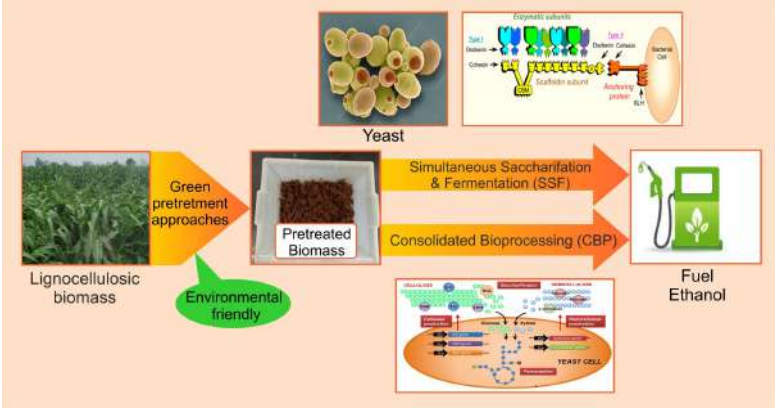
HIGHLIGHTS

- Recent advances in pretreatment of lignocellulosic biomass are reviewed and discussed.
- Use of green solvents, including ionic liquids and deep eutectic solvents, is presented.
- Strain improvement strategies to develop hyper-producing lignocellulolytic strains are compared.
- Advanced techniques for fermentation of mixed sugars in lignocellulosic hydrolysates are presented.
- Integration approaches for efficient biomass utilization and improved ethanol yields and productivity are discussed.

GRAPHICAL ABSTRACT



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ABSTRACT

Many countries have their biofuel policy programs in place as part of their overall strategy to achieve sustainable development. Among biofuels, bioethanol as a promising alternative to gasoline is of substantial interest. However, there is limited availability of a sufficient quantity of bioethanol to meet demands due to bottlenecks in the present technologies to convert non-edible feedstocks, including lignocelluloses. This review article presents and critically discusses the recent advances in the pretreatment of lignocellulosic biomass, with a 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 used in strain improvement strategies to develop hyper-producing deregulated lignocellulolytic strains are also compared and discussed. The advanced techniques employed for fermentation of mixed sugars contained in lignocellulosic hydrolysates for maximizing bioethanol production are summarized with an emphasis on pathway and transporters engineering for xylose assimilation. Further, the integration of different steps is suggested and discussed for efficient biomass utilization and improved ethanol yields and productivity.

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Contents

Abbreviations

[BMIM][$MeSO_3$]	1-butyl-3-methylimidazolium methanesulfonate	EMS	Ethanomethane sulphate
[EMIM][DEP]	1-ethyl-3-methylimidazolium diethyl phosphate	EP-PCR	Error-prone PCR
[emim][OAc]	1-ethyl-3-methylimidazolium acetate	FAD	Flavin-adenine dinucleotide
TBA][OH]	Tetrabutylammonium hydroxide	FAEs	Feruloyl esterases
2G	Second generation	FGRFS	Federal government renewable fuel standards
AA	Auxiliary activity	GAX	Glucuronoarabinoxylan
AFase	α-L-arabinofuranosidases	GHGs	Greenhouse gases
AXEs	Acetyl xylan esterases	GHs	Glycosyl hydrolases
bglR	β-glucosidase regulatory gene	*gls* 2α	glucosidase IIα subunit of gene
CADO	Consolidated alcohol dehydration and oligomerization	*gpd*	Glyceraldehyde-3-phosphate dehydrogenase gene
CAZymes	Carbohydrate active enzymes	gTME	Global transcription machinery engineering
CBH	Cellobiohydrolases (Exoglucanases)	HMF	5-hydroxymethylfurfural
CBMs	Carbohydrate binding modules	ILs	Ionic liquids
CBP	Consolidated bioprocessing	LCB	Lignocellulosic biomass
CCR	Carbon catabolite repressor	LiP	Lignin peroxidases
CDH	Cellobiose dehydrogenases	LMCOs	Laccase like multicopper oxidases
CEs	Carbohydrate esterases	LPMOs	Lytic polysaccharide monooxygenases
CFER	Cell-free extract reaction	MDF	Max-min driving force
CFP	Cotton filter powder	MNNG	N-methyl-N'nitro-N-nitrosoguanidine
CH_4	Methane	MnP	Manganese peroxidases
ChCl-G	Choline chloride-glycerol	N_2O	Nitrous oxide
ChCl-LA	Choline chloride-lactic acid	OPEFB	Oil palm empty fruit bunch
ChCl-U	Choline chloride-urea	PASC	Phosphoric acid swollen cellulose
CMC	Carboxymethyl cellulose	PCTR	Pilot-scale continuous tubular reactor
CO_2	Carbon dioxide	PEG	Polyethylene glycol
COVID-19	Corona virus disease	PPE	Personal protective equipment
cre1	Carbon repressor gene	rDNA	Recombinant DNA
CRISPR-Cas	Clustered Regularly Interspaced Short Palindromic Repeats	RNAi	RNA interference
DCR	Dirty cotton residue	SHF	Separate hydrolysis and fermentation
DES	Deep eutectic solvents	SiRNAs	Short-interference RNAs
EG	Endoglucanases	SSCF	Simultaneous saccharification and co-fermentation

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Abbreviations

SSF	Simultaneous saccharification and fermentation	XI	Xylose isomerase
$t_{1/2}$	Half-life	XK	Xylulokinase
VP	Versatile peroxidases	XR	Xylose reductase
WHO	World Health Organization	βG	β-glucosidases
XDH	Xylitol dehydrogenase		

1. Introduction

The ever-increasing demands for energy due to rapid increase of global population, industrialization, and geopolitical factors have called for the search for alternative and carbon neutral sources of energy (Souza et al., 2017; Chandel et al., 2020). For many years, the primary sources of energy have been non-renewable fossil fuels, oil, natural gas, and coal. However, these energy sources are inadequate to fulfil today`s most significant requirements of the societies in particular from the environmental and public health perspectives. More specifically, the widespread application of conventional energy resources has contributed to serious challenges, including global warming and climate change by releasing greenhouse gases (GHGs) like carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), and chlorofluorocarbons (Kiran et al., 2014). In fact, these adverse impacts have overshadowed the previous justifications used, including burgeoning petroleum prices, finite nature of fossil fuels, and have encouraged government and non-government agencies to find environmentally friendly, renewable, and sustainable energy resources for transportation, heating, and electricity generation (Nikolić et al., 2016).

Among these alternative energy carriers, ethanol has attracted a great deal of attention. It should be noted that ethanol is also widely used in a number of other industries and sectors among which the healthcare sector is largely highlighted due to the current COVID-19 pandemic and the recommendations by the World Health Organization (WHO) on the use of disinfectants containing alcohols like ethanol and isopropanol for adequate inactivation of coronavirus (Kratzel et al., 2020). This has for sure intensified the global demands for this valuable commodity and hence, a larger magnitude of production is required which in turns imposes more pressure on the already limited feedstocks available, i.e., first-generation feedstocks such as sugars and starch.

Given the above, lignocellulosic biomass (LCB) used as economic, widely-available non-edible feedstock for second-generation (2G) biofuels are highlighted more than ever (Kuila et al., 2016; Branco et al., 2019). If reaching their full potentials, LCB-derived liquid biofuels can cover approximately 40% of the total energy consumption of the world (Meher et al., 2006; Gielen et al., 2019). LCB of different origins such as banana plant waste (Ingale et al., 2014; Jahid et al., 2018; Khaliq et al., 2020), barley straw (Lara-Serrano et al., 2018), corn stover (Zakir et al., 2016; Dhiman et al., 2017), cotton stalk (Nikolić et al., 2016), and sugarcane bagasse (Wong and Sanggari, 2014; Zakir et al., 2016; Cheng et al., 2019) have been utilized for bioethanol production previously.

Three major steps are involved in LCB conversion into 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 their high availability and cost-effectiveness, the production of fuel ethanol and other high value-added products with high yield and productivity is 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 hydrolyze the polymeric cellulose and hemicellulose into fermentable monomeric sugars (hexoses and pentoses). Enzymatic hydrolysis is preferred over acid hydrolysis due to lower energy requirements 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 enzyme synergy and effectiveness (Myat and Ryu, 2016; Lugani and Sooch, 2018; Cheng et al., 2019; Kucharska et al., 2020). Following pretreatment and hydrolysis, fermentation of monomeric sugars is accomplished *via* microbial action to produce ethanol.

The selection of microorganisms for industrial bioethanol production depends upon their ability to utilize a wide range of substrates, being resistant against various inhibitory products, and tolerance to high sugar and ethanol concentrations (Hans et al., 2019). The yield and productivity of ethanol is much less with wild microbial strains, hence, developing genetically-modified microbial strains capable of meeting these requirements at industrial scale has been a primary focus. In light of these, the aim of the present article is to review and critically discuss the advanced approaches used for the pretreatment of LCB, 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 review articles published in last five years in this domain are tabulated in **Table 1**.

2. Pretreatment

As mentioned earlier, pretreatment is a necessary step to unwind the complex structure of LCB composing mainly of cellulose, hemicellulose, and lignin (Kassaye et al., 2017). A suitable pretreatment method is key in breaking down/redistribute the recalcitrant lignin structure leading to the accessibility of polysaccharides towards hydrolytic enzymes for their conversion into monosaccharides. In fact, an efficient pretreatment method largely facilitates the hydrolysis process leading to 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 which is at the same time convenient to perform, environment friendly, and economically feasible, is highly critical (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, physicochemical, and biological methods (Behera et al., 2014; Kumar and Sharma, 2017; Baruah et al., 2018; Gabhane et al., 2020; Hans et al., 2020). These are extensively studied methods but are associated with a variety of limitations such as low yield, high processing cost, and negative environmental impacts, and therefore, more efficient green technologies are being explored continuously to overcome these challenges (Capolupo and Faraco, 2016). **Figure 1** shows the different pretreatment approaches along with 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 associated with the conventionally used pretreatment methods for LCB, involving the use of hazardous chemicals. Ionic liquids (ILs)- and deep eutectic solvents (DES)-based pretreatments are among the most promising alternative methods owing to their ability to pretreat and selectively dissolve the constituents of biomass in a non-hazardous manner.

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Table 1.
Recent review articles (2015-2020) on bioethanol production through the utilization of lignocellulosic biomass.

S. No.	Review title	Coverage of review	Reference
1	Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: Concepts and recent developments	Different LCB as feedstock and their structural compositions; 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)
2	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)
3	Lignocellulosic biomass: A sustainable platform for the production of bio-based chemicals and polymers	Structure and sources of LCB; production of 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	Potential sources and composition of LCB; microorganisms and their lignolytic enzymes; overview on LCB conversion into bioethanol; 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 contained in LCB; fermentation of sugar to ethanol; pretreatment technologies for agricultural wastes along with advantages and disadvantages of each method	Nwosu-Obieogu et al. (2016)
7	Recent progresses in bioethanol production from lignocellulosic materials: A review	Lignocellulosic and algal feedstocks for bioethanol production; different pretreatment and hydrolysis methods for LCB; fermentation methods including integrated approaches (SHF, SSF, SSCF, SSFF, CBP); ethanol recovery; LCB biorefinery	Haq et al. (2016)
8	Bioethanol production from lignocellulosic waste- A review	Different pretreatment methods for agricultural wastes including wheat straw, rice straw, corn straw, and bagasse; enzymatic hydrolysis; fermentation	Mohanty and Abdullahi (2016)
9	A prospective of bioethanol production from biomass ass alternative fuel for spark ignition engine	Feedstocks used for different generations of biofuels; process for conversion of biomass to bioethanol; fuel properties of bioethanol; engine performance and emission characteristics 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 a special emphasis on SSF; yeast genetic engineering; ethanol production from different thermotolerant yeasts	Sharma et al. (2016b)
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; 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 inhibitor 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; 2G biofuel production from biomass using microbes; different approaches to enhance biofuel production; current production status of bioethanol	Bhatia et al. (2017)
14	Insight into progress in pretreatment of lignocellulosic biomass	An overview on biochemical routes including different processes for bioethanol production; challenges, advantages, and recent developments in different pretreatment processes; quantitative comparison 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 LCB; pretreatment methods with their action mechanisms for extraction of sugars (pentoses and hexoses) from LCB; existing challenges in pretreatment of LCB	Chen et al. (2017)
16	Recent updates on different methods of pretreatment of lignocellulosic feedstocks: A review	Composition of common LCB, different pretreatment processes along with 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 LCB 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 along with their advantages and disadvantages; sugar production from WH; fermentation and ethanol production considering the latest 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; commercialization of biomass to bioethanol processes	Awoyale and Lokhat (2019)
20	Recent trends in the pretreatment of lignocellulosic biomass for value-added products	Structure and composition of LCB; 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 improving lignocellulosic ethanol production; lignocellulose-derived platform chemicals	Bilal et al. (2018)

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Table 1.
Continued.

S. No.	Review title	Coverage of review	Reference
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 2G bioethanol through pretreatment, hydrolysis/ saccharification, fermentation, recovery, and dehydration; bioethanol production from kraft pulp, spent sulfite liquor, and paper and pulp sludge; 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 et al. (2019a)
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 LCB; conversion of LCB into ethanol using different types of pretreatment, enzymatic hydrolysis, and fermentation methods (SHF, SSF, SSCF, CBP); pentose fermentation and xylose metabolism; recombinant fermentative microbes; distillation and dehydration for ethanol recovery; latest 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 LCB along with the 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; advantages and disadvantages of various physical, physico-chemical, and biological pretreatment methods; challenges faced in pretreatment of LCB; integrated approaches for bioethanol production (SHF, SSF, SSCF, CBP)	Singh and Satapathy (2018)
30.	Bioethanol production from lignocellulosic biomass by environment-friendly pretreatment methods: A review	Composition of various types of LCB; pretreatment techniques for LCB; ethanol recovery from different feedstocks	Tayyab et al. (2018)
31.	Lignocellulosic biomass for bioethanol: An overview on pretreatment, hydrolysis and fermentation processes	Sources and composition of LCB; bioethanol production steps including pretreatment, hydrolysis, and fermentation; 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 impacts; opportunities to produce bio-based chemicals alongside cellulosic ethanol; technology readiness level (TRL) of chemicals which have reached commercial-scale production	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 enzymatic hydrolysis of LCB and the role of pretreatment; comparison of IL, concentrate phosphoric acid (CPA), and N-methylmorpholine-N-oxide (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 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 of bioethanol; 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 along with their action mechanisms; hydrolysis of polysaccharides contained in 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; processes 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 along with advantages and disadvantages of each process; effect of different 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 corncob; 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 LCB and their compositions; conventional and advanced pretreatment technologies with advantages and limitations of each method	Cheah et al. (2020)

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Table 1.
Continued.

S. No.	Review title	Coverage of review	Reference
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 processes and their limitations; emerging membrane-based processes for lignocellulosic bioethanol production; advanced membrane-based enzymatic saccharification and fermentation for bioethanol production; advanced SSFF strategy for lignocellulosic bioethanol production; advanced membrane separation processes for recovery of bioethanol; status of lignocellulosic bioethanol production at international and national levels; 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 LCB into ethanol; ethanol yield of different raw materials using different pretreatment combinations	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 lignocellulosic hydrolysates by various methods; enzymatic hydrolysis; fermentation; distillation and dehydration	Robak and Balcerek (2020)
43	Recent advances in bioethanol production from lignocelluloses: A comprehensive review with a focus on enzyme engineering and designer biocatalysts	Green pretreatment approaches including the use of ILs and DES for pretreatment of LCB; enzymatic saccharification of LCB; microbial sources of lignocellulolytic enzymes; strain improvement strategies to achieve hyper-producing lignocellulolytic strains; fermentation and integrated approaches for ethanol production; strain development for co-fermentation of xylose and glucose; pathways and genes involved in xylose metabolism; utilization of xylose through engineering the xylose isomerase pathway; engineering of transporters for improved xylose uptake	Present review

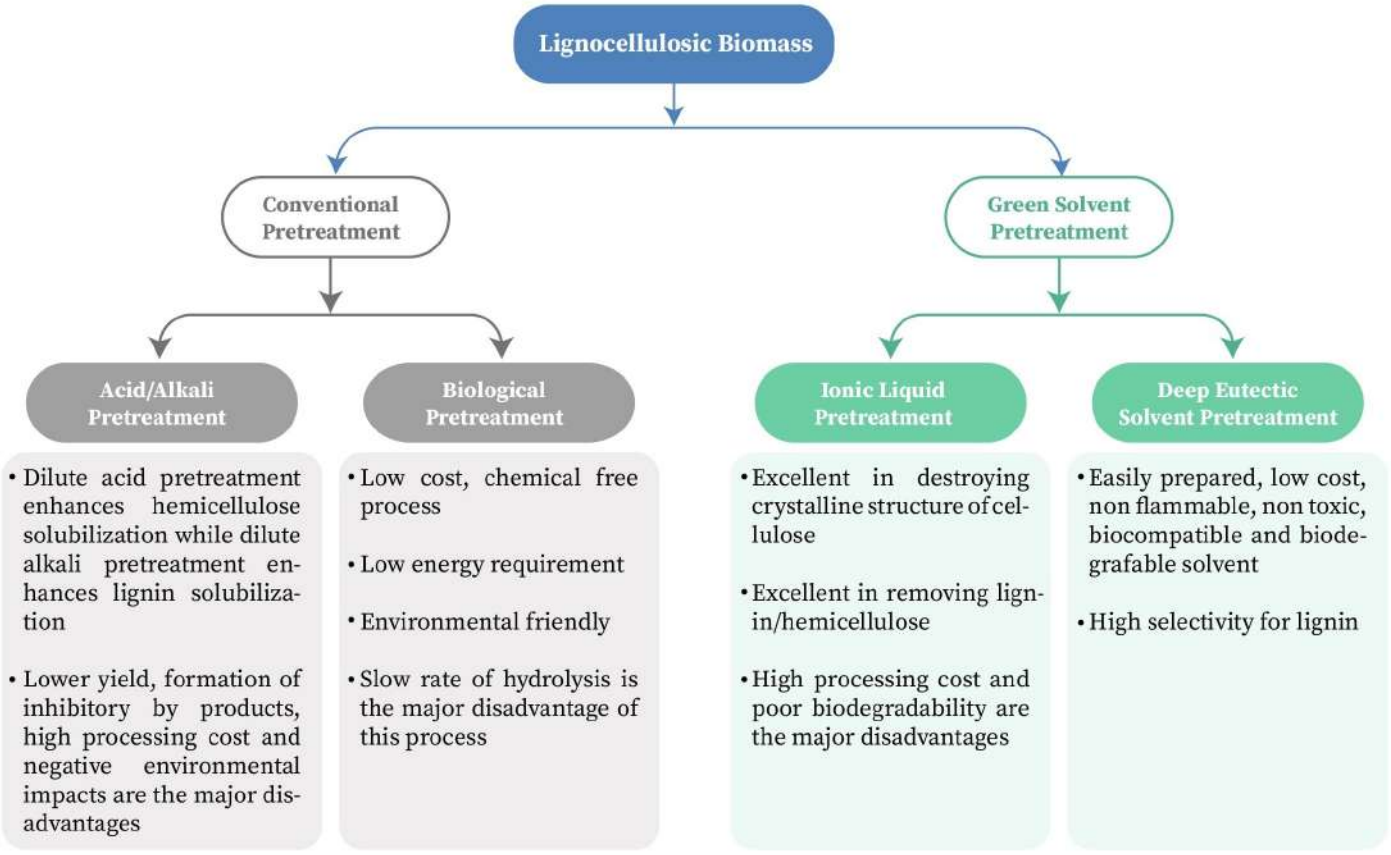

Fig. 1. Different pretreatment approaches, along with their pros and cons.

2.2.1. Ionic liquid pretreatment

ILs are known as "green solvents" due to their higher thermal and chemical stability, low vapor pressure, and non-flammable nature (Wu et al., 2014; Wahlström and Suurnäkki, 2015). ILs are liquids composed of ions with strong electrostatic bonding, making them less volatile, and electrochemically-stable (Socha et al., 2014). Moreover, ILs` characteristic could be designed by altering the combination of cations and anions. The cations contained in ILs are organic, while the anions could be either inorganic or organic (Brandt et al., 2011).

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Table 2.
A summary of the studies on the effects of ionic liquid pretreatment on LCB conversion.

S. No.	Biomass type	Ionic liquid	Pretreatment conditions	Effect on saccharification	Reference
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 by 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)

Many studies have revealed that ILs-pretreated LCB exhibited increased surface area with reduced lignin content and cellulose crystallinity (Wu et al., 2011; Li et al., 2010; Kassaye et al., 2017). 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 and glucose yields. Financie et al. (2016) observed that oil palm frond biomass pretreated with 1-ethyl-3-methylimidazolium diethyl phosphate [EMIM][DEP] at 110°C for 1 h showed an enhancement in cellulose content from 45.7% to 68.5.1% while a reduction of 8.5% and 12.1% was also observed in lignin and hemicellulose contents, respectively. Wang et al. (2018) investigated an ultrasound-assisted aqueous IL pretreatment approach (tetrabutylammonium hydroxide ([TBA][OH])) for eucalyptus. They argued that the pretreated sample showed a marked enhancement in the initial enzymatic rate of cellulose (79.39 mg/g/h) as compared to the untreated sample (17.63 mg/g/h). Recently, Fockink et al. (2020) observed the effect of 1-ethyl-3-methylimidazolium acetate [emim][OAc] during the pretreatment of dirty cotton residue (DCR) and cotton filter powder (CFP) at 140°C for 2 h. DCR yielded a high glucose yield of 78%, xylose yield of 94.9%, and delignification level of 45.5%, while CFP provided a 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 biorefineries (Hou et al., 2013).

2.2.2. Deep eutectic solvent pretreatment

Abbott et al. (2003) attracted the attention of the scientific community 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 biodegradability. The use of DESs has been explored as extraction solvents (García et al., 2016; Jenkin et al., 2016), reaction media, and electrolytes (Jhong et al., 2009; Alonso et al., 2016). These compounds have also been used extensively in recent years in the pretreatment of LCB for achieving high hydrolysis and fermentation yields as tabulated in **Table 3**.

Several studies have reported DES as a reasonable reaction media for enzymatic reactions as compared to conventional organic media (Gill and Vulfson, 1994; Erbeldinger et al., 1998). DESs are also promising solvents for dissolving a considerable proportion of the lignin contained in 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% lignin extraction. Pretreatment of rice straw with ChCl-LA was reported in a different study leading to a lignin dissolution of 68 ± 4 mg/g (Kumar et al., 2016). Solubilization of lignin and hemicellulose with cellulose digestibility of >90% have been reported when corn stover and corncobs were pretreated by using DESs (Xu et al., 2016; Zhang et al., 2016; Hou et al., 2017). Loow et al. (2018) reported that the 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 (pretreatment conditions: 150°C for 16 h), such as apple residues, potato peels, and brewer's spent grains (Procentese et al., 2018). In this study, ChCl-U pretreatment lowered the energy requirement by about 28% as compared to NaOH pretreatment.

The effect of three different DESs, namely, ChCl-LA, ChCl-U, and choline chloride-glycerol (ChCl-G), during the pretreatment of OPEFB at 120°C for 3 h with the solid-to-liquid ratio of 1:10 (w/v) was studied and compared to acid and alkaline solvents (Thi and Lee, 2019). 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 the cellulose fraction to enzymatic saccharification.

3. Enzymatic saccharification of lignocellulosic biomass

Conversion of LCB into pentose/hexose sugars with industrially desired yields is one of the major bottlenecks in production of 2G biofuels since there are several challenges associated with achieving high process

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Table 3.
A summary of the studies on the effects of DES pretreatment on LCB conversion.

Biomass	Deep eutectic solvent	Pretreatment conditions	Effect on saccharification	Reference
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 et al. (2019a)

efficacies. Acid hydrolysis is the most convenient and widely employed method for hydrolysis of polysaccharides into monomers *via* breakdown of hydrogen bonds 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 (Sun and Cheng, 2002; Harmsen et al., 2010; Binod et al., 2011; Al-Battashi et al., 2019). On the other hand, enzymatic saccharification of LCB is a suitable alternative as it is a mild and eco-friendly approach with lower energy requirements (Rai et al., 2016b; Al-Battashi et al., 2019; Ummalyma et al., 2019).

The enzymes required for efficient deconstruction of polysaccharides into monomeric sugars include 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 related to 2G ethanol production vary significantly, e.g., 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). Such an inconsistency in cost estimation hinders 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 (Sathya and Khan, 2014; Ezeilo et al., 2017). A total of 115 GH families comprising modular and non-modular cellulases and hemicellulases have been identified so far. These families of enzymes have been classified on the basis of their amino acid sequences and modes of action (Rai et al., 2016a and b; Ezeilo et al., 2017). The catalytic activity of GH family enzymes is achieved either through inversion or retention mechanisms resulting in products with a 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 **Figure 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) (Mandels and Weber, 1969; Bayer et al., 1998; 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). EGs have been reported from the 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 (Sweeney and Xu, 2012; Ezeilo et al., 2017). Among the identified families, the EGs belonging to GH 6, 9, and 48 follow the inversion mechanism, whereas, the rest act through the 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 subsequently processed by exoglucanases to form cellobiose (catalyzed by CBH) and/or glucose (catalyzed by βG) in a processive manner (Sweeney and Xu, 2012; Ezeilo et al., 2017). CBH 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 (Poidevin et al., 2013; Ezeilo et al., 2017; Patel et al., 2019). βG enzymes are broadly classified as members of GH family 1, 3, 5, 9, 30, and 116; however, the majority of the βG enzymes reported from fungi belong to the GH family 3 (Singhania et al., 2013).

Hemicellulases, another group of GHs, 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) (Juturu and Wu, 2013; Bhattacharya et al., 2015; Ezeilo et al., 2017). Among diverse hemicellulases, endoxylanases and β-xylosidases, collectively known as xylanases, are the most extensively studied enzymes. Endoxylanases have

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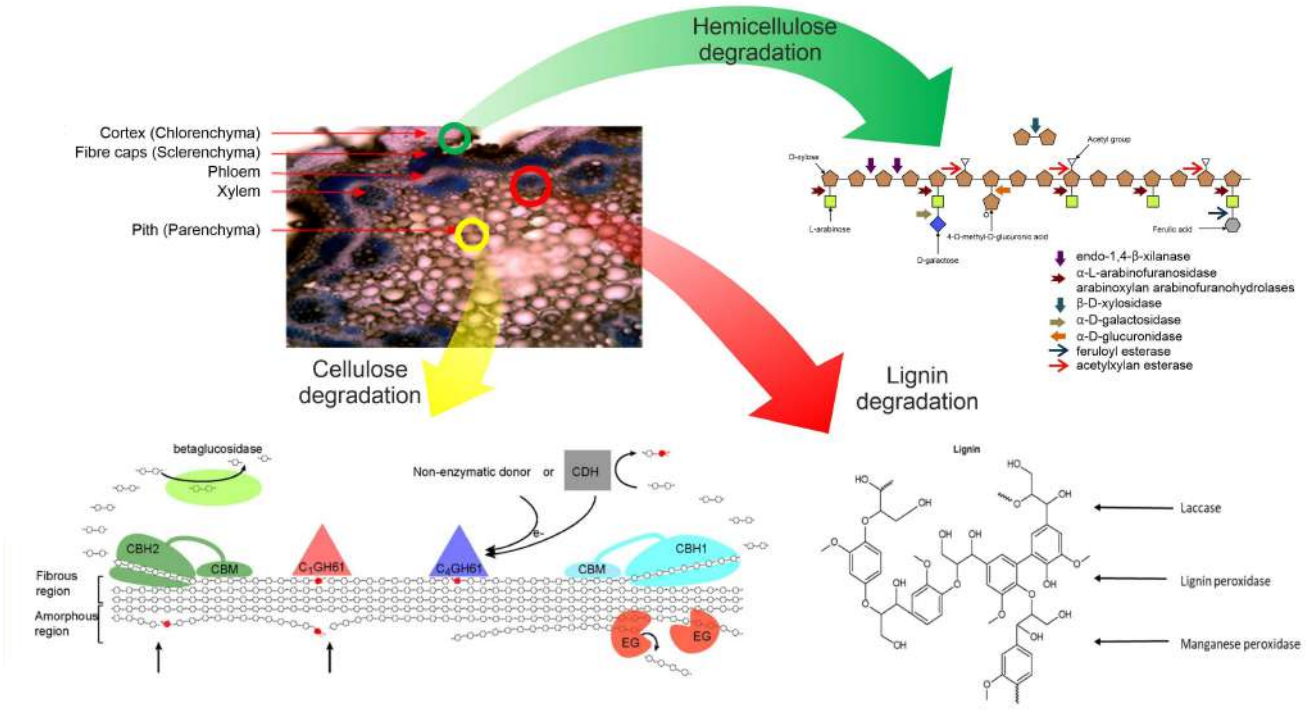

Fig. 2. Lignocellulolytic enzymes in action on plant cell wall.

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 (Lombard et al., 2014; Bhattacharya et al., 2015; Ezeilo et al., 2017). Endoxylanases belonging to GH family 8 and 43 operate through the inversion mechanism, whereas, those which belong to GH 5, 10, and 11 operate *via* the retention mechanism.

β-xylosidase, another essential component of hemicellulases, hydrolyze xylobiose and other short-chain xylo-oligosaccharides by attacking their β-1,4 linkages. The majority of the identified β-xylosidases have been reported from GH families 3, 39, 43, 52, and 54 (Shallom and Shoham, 2003; Knob and Carmona, 2009; Bhattacharya et al., 2015). Both inversion (GH 43) and retention (GH 3 and 54) mechanism of actions can be observed in β-xylosidases (Shallom and Shoham, 2003; Knob and Carmona, 2009). 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 analyzed for their enzymatic function is very low (0.6%) with acetyl xylan esterases (AXEs) and feruloyl esterases (FAEs) being the most studied enzymes (Ulaganathan et al., 2015; Nakamura et al., 2017). Both AXEs and FAEs are the hemicellulolytic esterases where the former group hydrolyse acetyl substitutions on xylose fractions, and the latter group mediate hydrolysis of ester linkages holding ferulic acid and arabinose substituents together (Ulaganathan et al., 2015; Nakamura et al., 2017).

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 AA 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 LCB (Levasseur et al., 2013; Ezeilo et al., 2017). 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 et al., 2019b). Laccase like multicopper oxidases (LMCOs; EC 1.10.3.2) constitutes another subfamily of AA1 proteins which shows involvement in delignification (Levasseur et al., 2013; Berni et al., 2019).

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

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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 (Levasseur et al., 2013; Janusz et al., 2017).

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 a major proportion of this family (Levasseur et al., 2013; Bodenheimer et al., 2018). 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 with the hydrolysis of LCB in recent years (Levasseur et al., 2013; Basotra et al., 2019). To 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; Walton and Davies, 2016; Müller et al., 2017). LPMOs require reducing equivalents to trigger their catalytic activity, and these equivalents are generated by functional electron donors like ascorbic acid or gallic acid (Vaaje-Kolstad et al., 2010; Quinlan et al., 2011) 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 (Wymelenberg et al., 2010; Phillips et al., 2011; Yakovlev et al., 2012; Rai et al., 2020). 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), has been recently 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 (Levasseur et al., 2013; Ezeilo et al., 2017). It has been 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 microbial lignocellulolytic enzymes (cellulase, hemicellulases, and auxiliary) have been reported from diverse ecological niches including forests, compost piles, composting soils, rumens, wood-processing plants, and sewage sludge (McDonald et al., 2012; Rai et al., 2016b; Patel et al., 2019). There are two most prominent lignocellulolytic systems: (i) extracellular enzymes in filamentous fungi and aerobic bacteria and (ii) enzyme complexes called as cellulosomes in anaerobic bacterial and fungal strains (Mathew et al., 2008; Arora et al., 2015b; Ezeilo et al., 2017). The complexed cellulolytic systems (cellulosomes) have been reported from several anaerobic microbes, e.g., *Acetivibrio*, *Bacteroides*, *Clostidium*, *Ruminococcus*, *Nocallimastix*, *Piromyces*, and *Orpinomyces* (Doi and Kosugi, 2004; Fontes and Gilbert, 2010; Sadhu and Maiti, 2013; Blumer-Schuette et al., 2014). The interaction of cellulosome with cellulose is shown in **Figure 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., and *Aureobasidium pullulans* LB 83 have exhibited significant cellulase, hemicellulase, and auxiliary activities (Sadhu and Maiti, 2013; Pang et al., 2017; Chadha et al., 2019; Islam and Roy, 2019; Rai et al., 2019b). *Bacteroides luti* and *Oricola cellulosilytica* are the examples of novel cellulolytic bacteria that have been isolated from methanogenic sludge and surface of seashore, respectively (Shinoda et al., 2012; Hameed et al., 2015; Chadha et al., 2019).

Although different microbial genera capable of producing lignocellulolytic enzymes, fungi have turned out to be the major role players (Marjamaa et al., 2013; Rai et al., 2016b; Patel et al., 2019). *Humicola grisea*, *H. insolens*, *Chaetomium thermophilum*, *Sporotrichum thermophila*, *Talaromyces emersonii*, *Myceliophthora thermophila*, *Thermoascus auranticus*, *Melanocarpus albomyces*, *Aspergillus* sp., *Trichoderma reesei*, *Acremonium cellulolyticus*, *Penicillium* sp., and *Trametes versicolor* are among the most potent lignocellulolytic fungi reported over the past couple of decades (Jatinder et al., 2006; Zambare and Christopher, 2010; Liu et al., 2013; Marjamaa et al., 2013; Phitsuwan et al., 2013; Rai et al., 2016b; Basotra et al., 2019; Sun et al., 2019). Among diverse lignocellulolytic fungi, *T. reesei* and *Aspergillus* sp. have been extensively exploited as lignocellulolytic strains at commercial scale (Gusakov, 2011; Chekushina et al., 2013; Rai et al., 2016b). However, recent trends have shown a paradigm shift towards other fungal strains, i.e., *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 scale 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 potent *Penicillium* strains that have been tested and validated for efficient degradation of different pretreated biomass (Huang et al., 2015; Yao et al., 2015; Rai et al., 2016b; Saini et al., 2016).

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, clustered regularly interspaced short palindromic repeats (CRISPR-Cas) system, and genome and metabolic engineering have been used in recent years to improve the enzymatic expression by microbial strains (Fujii et al., 2018; Basotra et al., 2019; Lim et al., 2019b; 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 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 the past decade (Chadha et al., 2005; Adsul et al., 2007; Fang et al., 2009; Liu et al., 2013; Kaur et al., 2014; Ottenheim et al., 2015; Lim et al., 2019b). 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 (Ilmén et al., 1996; Geysens et al., 2005; Nitta et al., 2012; Fujii et al., 2013). Fujii et al. (2013) reported the upregulation of cellulases and xylanase in *Acremonium cellulolyticus* by disruption of *creA* gene.

Site-directed mutagenesis is also a 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 (by up to 7.93 folds) in *Bacillus amyloliquefaciens* DL-3, and enhance alkaline tolerance of EGIII in *T. reesei* (Wang et al., 2005; Vu and Kim, 2012). A study has suggested that the product of *bgl2* gene catalyzes 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,

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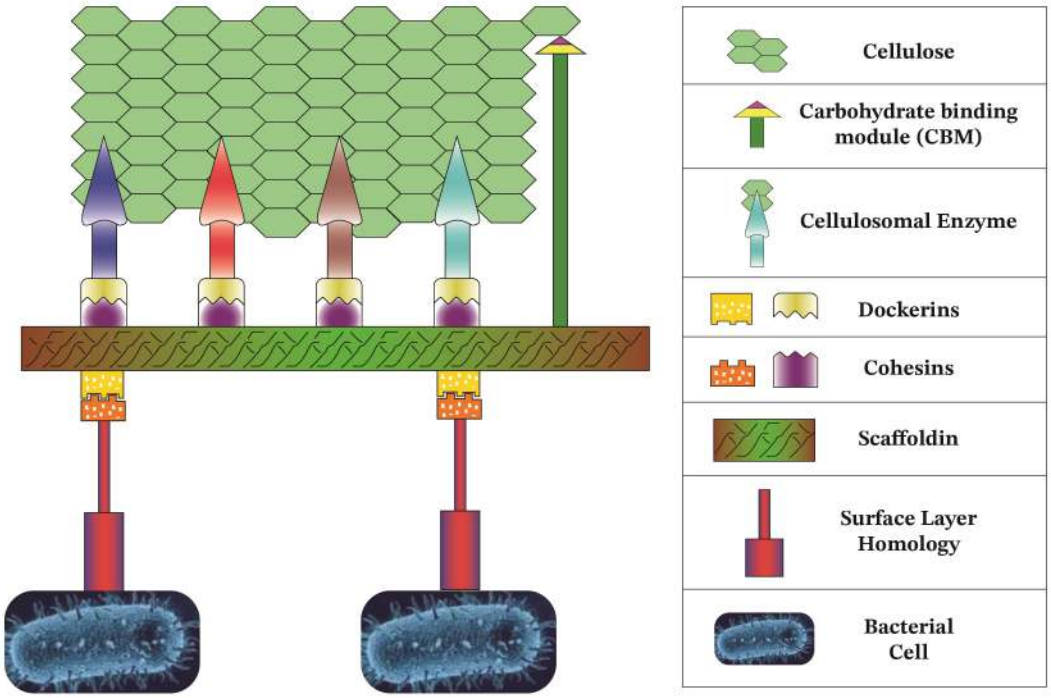

Fig. 3. Cellulosome in action for saccharification of cellulose. Modified from Arora et al. (2015b).

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 harboring genetic modifications. This technique is relatively inexpensive and simple, and shows enormous potential for developing industrially-competent hypercellulase strains without causing many disturbances in their physiology (Savitha et al., 2010; Kaur et al., 2013; Adeleye et al., 2019). 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 (Kordowska-Wiater et al., 2012; Kaur et al., 2013; Adeleye et al., 2019). 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 microspores* (Kordowska-Wiater et al., 2012), developing heterokaryons of two cellulolytic strains *Aspergillus tubingensis* and *A. nidulans* (Kaur et al., 2013), and improving amylase titers 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 study 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 to the 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).

Genetic engineering is also regarded 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 (Singh et al., 2017; Phillips, 2019). Methodologically, 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 (Singh et al., 2017; Basotra et al., 2019; Rai et al., 2019b). 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 (Poidevin et al., 2013; Fang and Xia, 2015; Basotra et al., 2019; Rai et al., 2019b). 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. In a different investigation, 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 hydrolyze 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

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(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 in *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 a 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 was capable of 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 on 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 the 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 and b; Rai et al., 2019b) and expressed in *E. coli* and/or in 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 (Gibney and Nolan, 2010; Mello-de-Sousa et al., 2016; Druzhinina and Kubicek, 2017). 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 domain 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 the application of the 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; Selim et al., 2018; Ding et al., 2020). 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 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, with intermittent additions 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 a constant addition of substrate and nutrients with continuous removal of products from bioreactor in the 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 labor-intensive process (Sanchez and Cardona, 2008; Kumar et al., 2015).

Techno-economic analysis of a pilot-scale production of bioethanol with high yields using *Z. mobilis*, revealed that ethanol production using this bacterium could save the cellulosic ethanol production facility by $2 million/yr (Kremer et al., 2015). Another study on pilot-scale production of bioethanol from dilute sulphuric acid-pretreated wheat straw by using recombinant *E. coli* FBR5 in a simultaneous saccharification and fermentation (SSF) system 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 an enhanced bioethanol production of 20.6 g/L with volumetric productivity of 1.0 g/L/h from food waste in a SSF system using the mixed culture of *F. oxysporum* F3 and *S. cerevisiae*. The supplementation of glucoamylase into the 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 maximum yield and productivity in bioethanol production, the selected microbial strain should have some unique features such as a broad range of substrate utilization, ability to withstand high concentrations of sugar, ethanol, 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 the alcoholic fermentation possess the ability to ferment hexose sugars only with very low ethanol yields and productivities. 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 their selection as industrially important, and hence, over the last few years, the focus has been placed on the development of genetically modified microorganisms to ensure their use in industrial applications.

4.1. Strain development for improved bioethanol fermentation

There are various previous studies, which have been done using adaptive evolution to create mutant strains, which are resistant to high temperatures, salt concentrations, acetic acid concentrations, freeze-thawing, pentose sugars, and various stress inducers (Wati et al., 1996; Stanley et al., 2010; Abreu-Cavalheiro and Monteiro, 2013; Sharma et al., 2016a and 2017; Choe et al., 2019). The powerful tools used to develop industrially important fermenting microbial strains to meet the demands 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).

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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 on accelerating the rate of reaction, shifting the existing metabolic pathway towards production of useful products, enhancing substrate specificity, and 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; Kricka et al., 2014; Sar et al., 2017; Ko et al., 2018). The recombinant bacterial strains named *E. coli* and *Klebsiella oxytoca* showed the ability to utilize a 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 kinds of stressors including ethanol. The mutant yeast strain also showed enhanced glucose consumption at low temperatures compared to wild strains (Yazawa et al., 2007). Both ethanol tolerance and fermentation capacity of sake yeast strains were enhanced by overexpression 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 microorganisms 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 a 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 in 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 nutrient requirements, and could tolerate high temperatures and low pH values (McEwen and Atsumi, 2012). Similarly, constitutive promoter substitution and xylose metabolic integration was done in *S. cerevisiae* for producing an 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 a CBP system (Li et al., 2017). The engineering of both feedstock as well as microorganism has reportedly resulted in an enhanced bioethanol production. This strategy also provided the feasibility of ethanol production at commercial scale using lignocellulosic waste materials (Ko et al., 2018).

Safe and stable expression of cellulase gene (*sestc*) and glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) promoter was achieved using the 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 the mutant strain was observed to be stable for 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 into large scale bioethanol production.

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

The conversion of LCB into ethanol is associated with 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, only a few strains such as *S. shehatae*, *S. stipitis*, and *K. marxianus* have the ability to assimilate pentose sugars but the production of ethanol is not up to the industrial standards, and hence the co-fermentation of pentose and hexose sugars is a major obstacle in efficient conversion of LCB to ethanol (Kim et al., 2012; Arora et al., 2015c; Moysés et al., 2016).

For instance, among the organisms capable of converting sugars into ethanol, *S. cerevisiae* is the most widely used at the industrial scale due to its versatile characteristics such as high tolerance to ethanol, ability to withstand low pH values, ability to ferment under anaerobic condition, tolerance to high osmotic pressures, and less prone to bacteriophage infections (Robak and Balcerek, 2018). However, the yeast *S. cerevisiae* exhibits weak expression of pentose pathway gene, and have poor/no xylose uptake ability.

Over the last few years, the considerable developments in genetic engineering has changed the metabolic engineering paradigm. Specialized tool boxes are currently available for pathway manipulation of microbial 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 microorganisms during xylose metabolism through two different pathways. In filamentous yeasts, the oxidoreductase pathway having two-step reaction is involved. In the initial step, 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, 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 by xylulokinase (XK) (EC 2.7.1.17) to xylulose-5-phosphate, which is an intermediate for the phosphoketolase and non-oxidative pentose pathways. The pentose pathway can be further classified into two distinct pathways, namely non-oxidative and oxidative pentose phosphate pathways. Most of the yeasts use the non-oxidative pathway to metabolize xylulose-5-phosphate as a precursor for nucleic acid and amino acids production and also convert it to three, four, five, six, and seven atom carbon sources, which serves as intermediate to glycolysis. While the oxidative pathway is used as a defensive mechanism against oxidative stress and to generate NADPH, which is a major precursor for biomass formation, and is also a 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 as a result, the microorganism 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 the 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, respectively, cloned them in *S. cerevisiae* for the first time, and showed oxidative utilization of xylose. Later, more work on expressing *xyl1* and *xyl2* genes from *S. stipitis* in *S. cerevisiae* was carried out (Ho et al., 1998; Toivari et al., 2001; Kim et al., 2013), and reported higher assimilation of xylose and ethanol production as compared to the engineered strain

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overexpressed with XI gene (Karhumaa et al., 2007; Li et al., 2016). The XR-XDH is an oxidoreductases-based enzyme, and requires a 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 an excess accumulation of NADPH and reduced NAD^+ availability. Further the XR in *P. stipitis* has a higher affinity for NADPH (K_m= 3.2 µmol/L) as compared to NADH (K_m=40 µmol/L) and XDH completely relies on NAD^+, which causes severe disparity in the redox balance, leading to excess xylitol 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 the overexpression of the gene encoding furaldehyde reductase as a co-factor used in HMF reduction, significantly influenced ethanol production. They reported that the NADH-dependent reductase exhibited carbon conservation by reducing glycerol formation and enhancing NAD^+ availability for XDH, which eased 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. Two glutamate dehydrogenases namely NADPH-dependent glutamate dehydrogenase and NADH dependent glutamate dehydrogenase are responsible for ammonium assimilation in *S. cerevisiae*. Co-factor imbalance in the recombinant *S. cerevisiae* can be reduced by modifying ammonium assimilation through the 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). This strategy was reported to improve ethanol yield from 0.43 to 0.51 cmol/cmol while reducing xylitol accumulation by 44% (Roca et al., 2003). The increase in ethanol production and reduced xylitol production was due to the increase in the NAD^+ availability for XDH, which directed 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 ethanol yield was increased by 25% (Sonderegger et al., 2004).

The accumulation of xylitol was reduced when glucose was used as a co-substrate; however, this costs in reduced xylose assimilation due to the competition among the sugar transporters (Hallborn et al., 1994). A recombinant *S. cerevisiae* strain with an XR to XDH ratio of 0.06 showed no xylitol and acetic acid formation, and depicted a good ethanol yield as compared to the strain with a higher XR:XDH ratio (Walfridsson et al., 1997). Multicopy integration of *xyl2* gene encoding XDH in the recombinant *S. cerevisiae* strain elevated xylulose accumulation and reduced 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 reduce intracellular levels of ATP and the ATP/ADP ratio with the subsequent overexpression of XR, XDH, and XK substantially enhancing 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 an ethanol yield of 0.47 g/g emphasizing on the role of XR in increasing 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, it does not require co-factor and convert xylose directly to xylulose. Most of the heterologous expression of XI in *S. cerevisiae* strain shows lower functionality; probably 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 a 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 a high specific activity (1.0 U/ mg of protein) at 80°C but had a poor performance (with a 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 *T. 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 ethanol productivity and reduced xylitol production. The bottleneck related to xylitol accumulation and increasing 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 could 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 a K_m= 66.7 mM and specific activity of 1.41 µmol/min/mg. The recombinant *S. cerevisiae* harboring 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 could be increased by overexpressing the heterologous sugar transporter (*PsSUT1*) and *xk* in the engineered strain containing *xylA* from the fungus *Orpinomyces*, showing an ethanol yield of 0.48 g/g and low xylitol yield of 0.04 g/g when grown in a 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 an 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 a 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 efficient performance of the XR and XDH. Since NADPH co-factor is majorly generated through the oxidative pentose phosphate pathway, the 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 a low XR/XDH ratio. However, the mutant having *Δzwf1* and *Δgnd1* also showed reduced growth rate due to a 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) was performed which 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* led to a 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 co-factor imbalance on the production of by-products, i.e., ethanol and xylitol (Zhang et al., 2012). The

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Table 4.
A summary of the studies performed on the over-expression of heterologous pentose phosphate pathway genes for improving 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)	Reference
Saccharomyces 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

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 a limited exogenous xylose metabolism and a low affinity for xylose 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 et al., 2018a). The strength of the xylose transporter can be improved by targeting and engineering the existing sugar transporters or searching for novel heterologous xylose affinity/glucose repressor-based transporters (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 xylose uptake in *S. cerevisiae*; however, these transporters have low affinities towards xylose in the presence of glucose and are inefficient in xylose transportation at lower concentrations (Hamacher et al., 2002; Saloheimo et al., 2007). Among the hexose transporters, Hxt7 and Gal2 show higher affinities for xylose, but in the presence of glucose, these transporters are repressed and xylose uptake rate is reduced.

To enhance the uptake of xylose in *S. cerevisiae*, Leandro and co-workers expressed *C. intermedia* PYCC 4715 transporter proteins (glucose/xylose symporter - Gxs1 and glucose/xylose facilitator - Gxf1) in *S. cerevisiae*. The recombinant strain exhibited a higher growth rate in a xylose-containing medium with K_m=0.2 mM, 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 a hexose null mutant which barely showed any improvements on a xylose-containing medium. When Gxf1 was expressed in *S. cerevisiae*, the recombinant strain showed a higher xylose uptake at lower concentrations of xylose, but it was unchanged even at higher concentrations of xylose in aerobic condition. The strain also exhibited a 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 the directed evolution method and expressed these mutant genes in a hexose-null *S. cerevisiae* mutant. The recombinant strain showed a substantial growth and uptake of xylose in a 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 a higher xylose uptake ability with V_{max}=186.4 nmol/mL/min as compared to the 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 a 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* leads to a higher affinity towards glucose than xylose; however, Sut1 has a higher V_{max} for xylose as compared to the other transporters. Over-expression of *P. stipitis* Sut1 in a recombinant *S. cerevisiae* harboring XR-XDH-XK genes showed a 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 a hexose-null mutant strain (*hxt1Δ-hxt7Δ* and *gal2Δ*) harboring *xyl1*, *xyl2*, and *xk* genes. The results revealed that Hxt7 had a higher xylose consumption ability compared to the other transporters; however, the substrate affinity was 200 folds higher for glucose as compared to xylose in the medium containing glucose/xylose mixture making xylose the second choice even in the presence of low concentrations of glucose. While Hxt1 showed higher sugar uptake and ethanol productivity in co-fermentation of glucose and xylose but severely repressed xylose uptake in the presence of glucose showing diauxic growth profile. To overcome the barrier related to transporter repression, 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 it contributed to the understanding of sugar-transport mechanisms. More specifically, single point mutation in N376-F region of Gal2 and N370-S region of Hxt7 led to higher affinity towards xylose and loss of ability to transport hexose sugars.

Nijland et al. (2017) adapted an evolutionary engineering strategy to develop a chimeric HXT36 by the fusion of functional hexose transporter Hxt3-Hxt6. An amino acid substitution at N367A of Hxt36 enabled the co-consumption of glucose and xylose. The genome sequence analysis showed that co-repressors such as CYC8 and SSN6 were responsible for phenotypic characteristics of the non-evolved strain. Inactivation of CYC8 showed a higher activity of Hxt, which in turn increased the xylose transport and led to less sensitivity 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 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 a glucose and xylose containing medium. An overview of metabolic engineering in yeast for simultaneous uptake of glucose and xylose is depicted in **Figure 4**.

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

In microbial fermentation, the production of by-products is inevitable, which in turn diverts the carbon flux from the main product, 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

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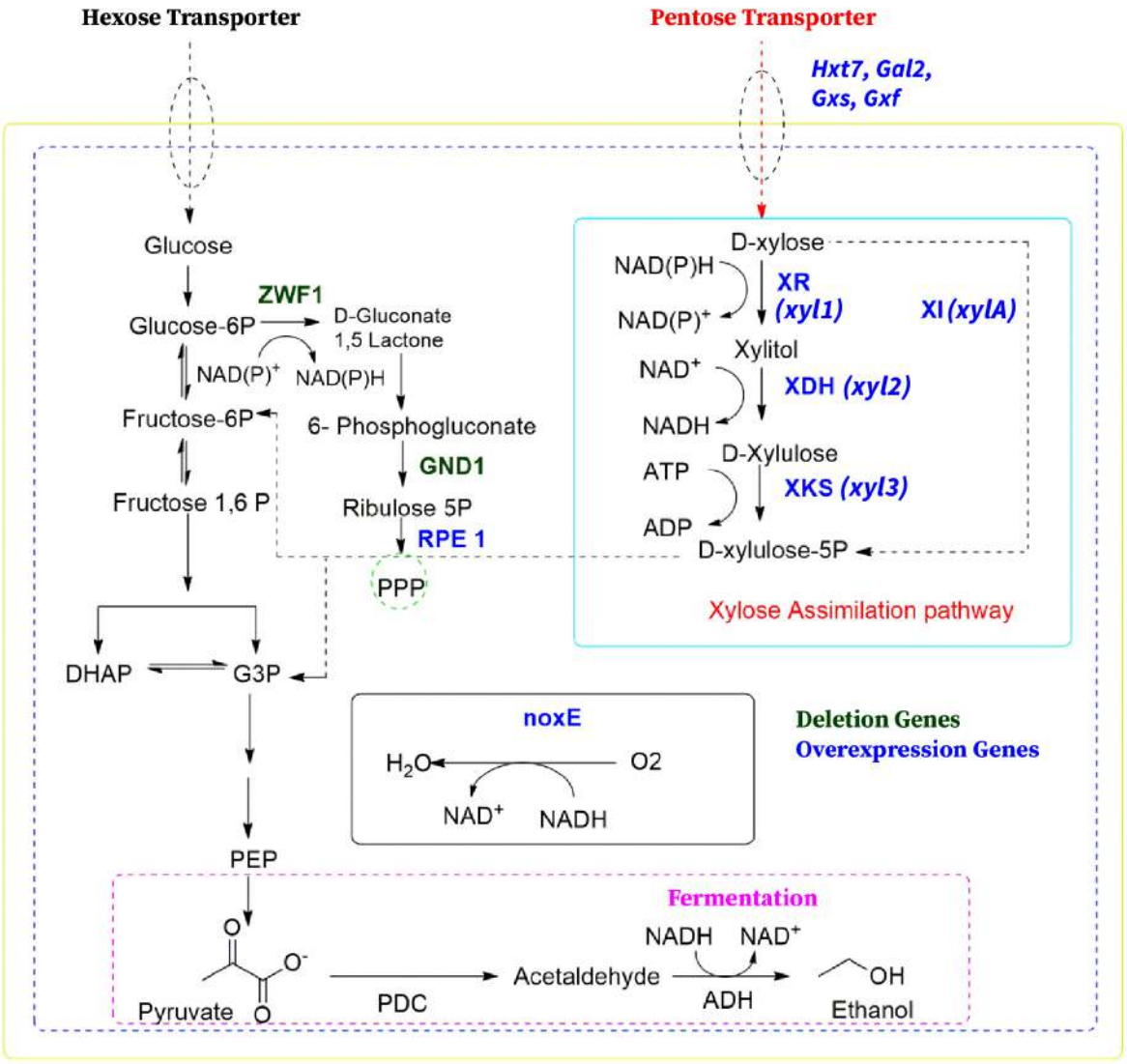

Fig. 4. Metabolic Pathway engineering for xylose assimilation in *Saccharomyces cerevisiae*. Dark Green represents the gene deletion and Blue represents the heterologous overexpression of genes. (XR: Xylose reductase; XDH: Xylitol dehydrogenase; XKS: Xylulose kinase; XI: Xylose isomerase; ZWF1: glucose-6-phsophate 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; Hxt7, Gal2, Gxs, and Gxf are transporters).

for 2-3% of sugar bioconversion. Even though glycerol is one of the platform chemicals, its separation during ethanol fermentation is not economically viable (Prior and Hohmann, 1997). In *S. cerevisiae*, 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 converts NADH to NAD^+ by production of sorbitol and propane-1,2-diol) and ethanol yield was maintained at 0.48 g/g glucose. On 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 a high osmolarity and through the consumption of acetic acid without producing glycerol.

Acetic acid is another product usually observed in *S. cerevisiae* fermentation. It 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 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* genes 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.

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5. Integration approaches

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, each process is optimized 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 increases 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 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 properties (titre and yield), and expression of heterologous cellulase system for cellulose utilization by high product-yielding non-cellulolytic microbes (Lynd et al., 2005).

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 offer easy process design and short reaction time, and are easy to operate with reduced process cost. However, there are also some limitations associated with these processes. One of the major challenges is the 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 30-35ºC to accommodate both microbial growth and ethanol fermentation (Canilha et al., 2012; Nikolić et al., 2016; Azhar et al., 2017). 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 the cellulosic part before fermentation. This results in difficulty in heat and mass transfer, and homogenous mixing of culture and media

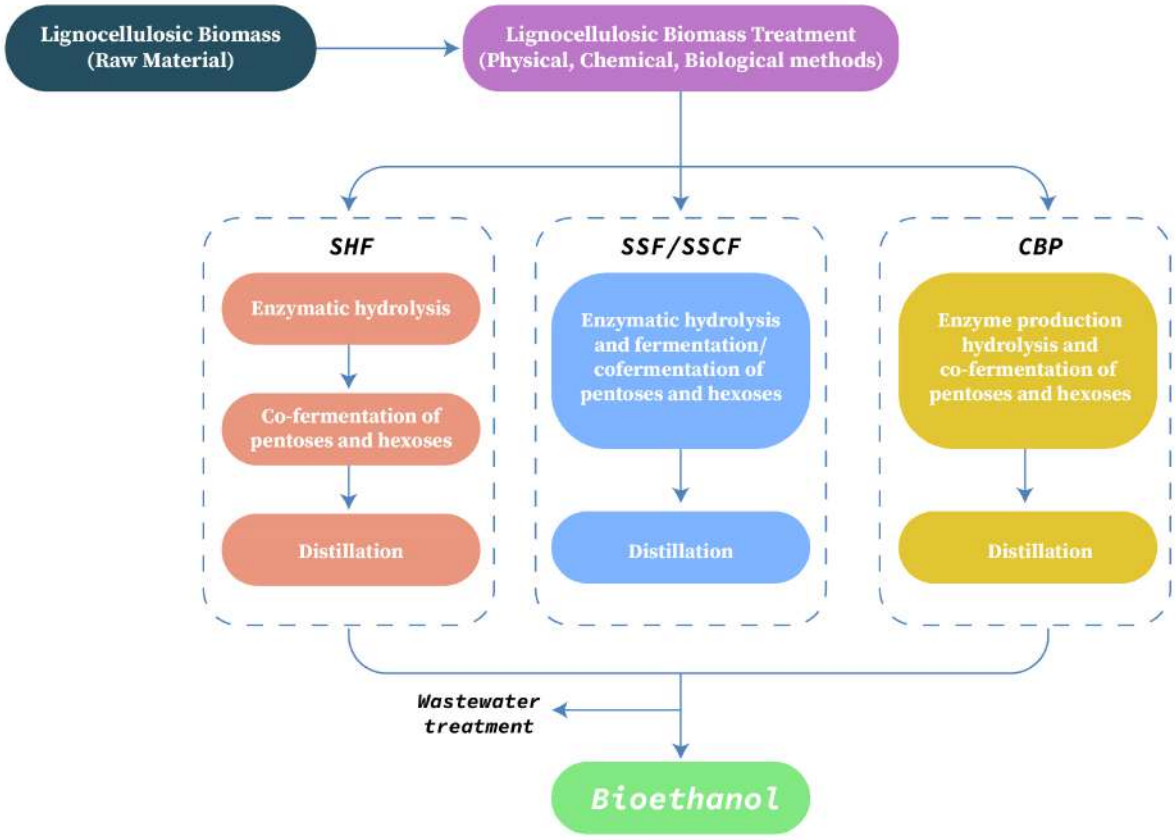

Fig. 5. Integration approaches for commercial production of bioethanol (SHF: Separate hydrolysis and fermentation; SSCF: Simultaneous saccharification and fermentation; SSCF: Simultaneous saccharification and co-fermentation; CBP: Consolidated bioprocessing).

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 a 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

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 (80 W for 5 min) of cornmeal (cornmeal to water ratio

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of 1:3) increased 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 a fed-batch mode with 25% (w/w) substrate loading and achieved an ethanol concentration of 30 g/L (Ballesteros et al., 2010).

In a study, the thermotolerant yeast strain *S. cerevisiae* KNU5377 was used for ethanol production from pretreated waste newspaper (250 g/L, solid loading) in a SSF system, and the ethanol production of 8.4% (v/v) was obtained at 50ºC after 72 h in a 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% of the substrate was converted into bioethanol at the end of fermentation with a product yield of 0.51 g/g sugar (Taouda et al., 2017).

In a more recent study, bioethanol production of 82.1 g/L was reported using sulphite-pretreated momentary pine slurry (25%, w/w) in a 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 also been reported on bioethanol production using SSCF fermentation (Erdei et al., 2013; Liu and Chen, 2016; Qin et al., 2018; Sharma et al., 2018b). SSCF improved ethanol production and productivity from food waste using *S. coreanus* and *P. stipitis*, standing at 48.63 g/L and 2.03 g/L/h, respectively (Jeong et al., 2012).

5.2. Consolidated bioprocessing (CBP)

The concept of 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 contributions to developing CBP systems for industrial ethanol production with high yields and titers from native and recombinant microbial strains (Laser et al., 2002; Lynd et al., 2005; Van Zyl et al., 2007; Olson et al., 2012; Holwerda et al., 2014; Izquierdo et al., 2014; Tian et al., 2017; Hon et al., 2018). In CBP, rational designs are used for production of engineered strains of bacteria and yeast with high ethanol titers (Jin et al., 2011). Filamentous fungi, *Fusarium oxysporum*, possesses the potential of bioethanol production from lignocelluloses in the 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 the CBP approach due to their ability for direct conversion of cheaper biomass feedstocks into bioethanol at extreme temperatures. 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 using the CBP platform has been reported by Raftery and Karim (2017). In another study, pine needle biomass was pretreated using IL followed by fermentation using *S. cerevisiae* and *P. stipitis* in a CBP system. The ethanol yield of 0.148 g/g was 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 (harboring integrated *temA* and *temG Opt* gene cassettes) simultaneously expressing α-amylase and glucoamylase, produced 89.35 g/L and 98.13 g/L ethanol from starchy biomass in a single step CBP system at 30°C after 192 h with carbon conversion of 87% and 94%, respectively (Cripwell et al., 2019).

Beri et al. (2020) proved the consumption of 85% recalcitrant glucuronoarabinoxylan (GAX) contained in from corn fiber by the isolated *Herbinix* spp. strain LL1355, and reported that six enzymes were involved in the hydrolysis of GAX linkages. They argued that the successful expression of up to four genes in *Thermoanaerobacterium thermosaccharolyticum* increased the GAX consumption and ethanol yield by 78% and 28%, respectively. Recently, the cell-free extract reaction (CFER) system was developed in *Clostridium thermocellum* to identify potential metabolic limitations and to offer potential metabolic engineering interventions to enhance ethanol titers (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 are some gaps concerning the commercial use of CBP systems. Future studies should be directed towards understanding the metabolic pathways of microorganisms, synergistic action between microbes and their enzymes for simultaneous pretreatment, hydrolysis and fermentation, and developing recombinant strains and bio-design strategies for enhanced ethanol production with improved yields.

6. Concluding remarks and future prospects

Lignocellulosic or 2G ethanol is being considered as one of the long-term sustainable alternative to the environmentally-degrading crude oil reserves. However, there are several technical and economic challenges associated with 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. The choice of pretreatment method relies on the type of biomass selected for 2G sugars production at competitive prices. Thermo-mechanical extrusion method is considered one of the most efficient pretreatment methods 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.

High cost associated with the commercially available cellulase/hemicellulase enzymes is another bottleneck that should 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. 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 future research should also target designing integrated approaches for simultaneous pretreatment and 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. 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. Therefore, the future research should be focused on the development of robust engineered yeast having suitable transporters for simultaneous uptake of glucose and xylose with equal assimilation rates. Metabolic engineering, cell surface engineering, and synthetic biology are other promising approaches 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. Among the various production platforms, CBP seems more efficient for economic bioethanol production because all the steps are performed in a single reactor by a single microorganism capable of producing hydrolytic enzymes and fermentation.

Finally, development of biorefineries seems critical for economical utilization of LCB. Focusing on a single product or bioethanol may not be an economically viable option. A biorefinery may be designed in such a fashion to valorize each and every component of lignocelluloses into biofuels and biochemicals for sustainable development of circular bioeconomy (Chandel et al., 2020). Therefore, a major focus should be placed on the development of such kinds of systems to reduce production cost and improve production efficiency.

Addressing the above-mentioned challenges could help to provide solutions for escalating global energy demands while mitigating the climate-related challenges as well.

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