Biorefinery Approaches For Production Of Cellulosic Ethanol Fuel Using Recombinant Engineered Microorganisms

Malinee Sriariyanun[1*], Marttin Paulraj Gundupalli[1], Vanarat Phakeenuya[2], Theerawut Phusamtisampan[2], Yu-Shen Cheng[3], and Ponnusami Venkatachalam[4]

[1] *Biorefinery and Process Automation Engineering Center, Department of Chemical and Process Engineering, The Sirindhorn International Thai-German Graduate School of Engineering, King Mongkut's University of Technology North Bangkok, Bangkok, Thailand*
[2] *Department of Biotechnology, Faculty of Applied Science, King Mongkut's University of Technology North Bangkok, Bangkok, Thailand*
[3] *Department of Chemical and Materials Engineering, National Yunlin University of Science and Technology, Douliu, Yunlin 64002, Taiwan*
[4] *Bioprocess Intensification Laboratory, Center for Bioenergy, School of Chemical & Biotechnology, SASTRA Deemed University, Thirumalaisamudram, Thanjavur 613401, India*
[*] *Corresponding author. E-mail: macintous@gmail.com*

Received: Nov. 13, 2022; Accepted: Mar. 08, 2023

Cellulosic ethanol has been gaining high attention due to its potential to reduce the greenhouse gas emission and cut down the world dependence on fossil fuels. Biorefinery approach for cellulosic ethanol has advantages due to its non-food competing status, natural abundance and benefit to decrease the combustion of agricultural wastes after harvesting seasons. Due to the recalcitrant structure of lignocellulose biomass, pretreatment and hydrolysis are critical to determine the economic viability of the process because they influence the conversion rate of fermentable sugars and, subsequently, final product i.e. ethanol. Therefore, the design for the process to compromise fermentation and upstream process is also essential. With all constraints exist when using harsh conditions during pretreatment, the recombinant engineered microorganisms have been developed and applied as biocatalysts during fermentation. To achieve the maximum production efficiency, different strategies of recombinant engineered microbes include expression optimization to modify the metabolic pathway, modification of secretion and transportation routes, improvement of stress tolerance, and utilization of both C5 and C6 sugars. This review provides the development and current status of cellulosic ethanol production via biorefining process by genetic engineered microbes with a focus on the technological aspects. The remaining challenges, perspective, and economical feasibility of the process are also discussed.

Keywords: Biorefinery, Enzymatic saccharification, Ethanol, Fermentation, Lignocellulosic biomass, Pretreatment, Recombinant engineering

http://dx.doi.org/10.6180/jase.202402_27(2).0001

1. Introduction

Ethanol production from lignocellulosic biomass is one of the most promising alternative to conventional transportation fuels and industrial solvents [1]. Lignocellulose ethanol gains interest due to two main reasons; the fluctuations of world market's price of fossil fuels and the awareness to global warming situation. Since 1999, the crude oil price steadily increased from 12.57 USD/barrel to the peak at 139.81 USD/barrel during subprime crisis

in 2008. Right after that in 2009, due to global recession, the crude oil price dropped to 41.4 USD (70.4% decreased in 1 years). The uptrend circle of price was returned the peaks at 98.6 USD/barrel in 2014, and it entered the downtrend again due to global economic recession in 2016 at 28.9 USD/barrel (70.7% decreased in 2 years) [2]. Due to the COVID-19 pandemic situation, the energy demand is rising within short time periods (during 2020 - 2022).

When the normal life and economic situation resumes from the global lockdown policy, it causes the price to rise from 20.9 to 123.7 USD/barrel (591.9% increase in 2 years). This situation presents the high risk of crude oil price and insecurity of energy supply leading to requirement to find sustainable energy source. On the other hand, due to the awareness of serious global warming situation, the 26th United Nations Climate Conference (COP26) was held in 2021 and the Sustainable Development Goals (SDGs) are set to achieve the global net zero emission by 2050 [3]. Lignocellulose biomass offers the benefit as non-food status, inertness, abundancy and sustainable raw material. Currently, several commercial processes for production of cellulosic ethanol are in operation worldwide, such as USA, China, Brazil, Romania, Japan, Austria, India, and Finland with the scale of production of about 30 billion gallons per year in 2020 [4].

Lignocellulosic biomass is composed of three polymer layers, including cellulose, hemicellulose, and lignin. The proportion of each polymer is varied depending on the type of lignocellulose biomass Table 1. Naturally, the physical and chemical properties of lignocellulose are recalcitrant to protect the invasion of phytopathogen and environmental stress. Thus, breaking down process, or hydrolysis, of lignocellulose polymers to monomers that are subsequently converted to ethanol is ineffective [5]. The conversion of lignocellulosic biomass to ethanol is usually conducted by a multi-step biorefining process, which is composed of pretreatment, hydrolysis, fermentation and product recovery and separation Fig. 1. The first step in pretreatment is to handle the bulky lignocellulose biomass from size reduction of raw materials to handling size by physical methods, such as cutting and milling. Then, the lignocellulose is pretreated by various methods that can be categorized as chemical, physical and biological methods. Pretreatment promotes the dissociations of lignocellulose fibrils, the removal of lignin (inhibitor), and modification of cellulose structure to be more accessible to cellulase enzyme [6]. After lignocellulose fibrils are pretreated, the biomass is hydrolyzed by cellulase enzyme or acid to liberate C6 (hexose) and C5 (pentose) sugars. The reducing sugars are converted to targeted products, including ethanol, biochemical and platform chemical by the catalysis of microorganisms (fermentation), enzymes or via thermo-chemical conversion process. The products are recovered, separated, and polished to be the final value-added products, such as biofuel, platform chemicals, biopolymers and biochemicals [7] (Fig. 1).

Based on the process flow of biorefining process, each step is continuously operated. Therefore, using harsh condition (e.g. extreme pH and temperature) in pretreatment could lead to the unsuitable condition for hydrolysis and fermentation. Various classes of inhibitory compounds are produced during pretreatment, including furan aldehydes (furfural and 5-HMF), weak acids (acetic acid, formic acid, levulinic acid), and phenolic compounds (vanillin, p-coumaric acid) [8]. These inhibitory compounds reduce the efficiency of microbial fermentation or impair the microbial survival rate. Hence, it has been a challenge to obtain high fermentation efficiency using wild strains of microorganisms in the presence of the inhibitors and unsuitable operational conditions [9]. On the other hand, in case of ethanol fermentation, *Saccharomyces cerevisiae* is one of the main microorganism that functions as a biocatalyst to convert hexose sugars to ethanol, however, it has less capability to utilize pentose sugars derived from hemicellulose (via pentose phosphate pathway) [10]. It is demonstrated that glucose acts as a barrier for the utilization of the xylose during fermentation process. Additionally, during the ethanol fermentation, the ethanol concentration could be accumulated up to 35-45 g/L in the reactor, which this much concentration can inactivate the functions of wild type microorganism [11].

To overcome these challenges of biorefining process for production of cellulose ethanol, the genetic engineered microorganism strains, especially *S.cerevisiae*, have been developed and implemented in commercial facility, such as Dupont VERBIO (USA) and Granbio Bioflex 1 (Brazil). In case of Granbio's facility, the recombinant *S.cerevisiae* is designed to be able to consume glucose and xylose obtained from cellulose and hemicellulose and this strain has been certified for commercial use by National Biosecurity Commission (CTNBio) since 2016 [8]. Another commercial strain of *S.cerevisiae*, CelluXTM is developed for cellulosic ethanol industry with ability to resist the stress, high ethanol tolerance and high viability in cellulose hydrolysate [12]. This review focused on the design and advanced techniques of biorefining process for cellulosic ethanol production. The strategies to improve the process efficiency by using genetic engineered microorganisms were showcased and their current situation and future direction were discussed to provide the idea for further development for

Table 1. Different types of lignocellulosic biomass utilized as feedstock for the production of ethanol

Lignocellulosic biomass	Cellulose, %	Hemicellulose, %	Lignin, %
Corncob	50.5	31	15
Rice straw	32.1	24	18
Tea waste	30.20	19.9	40
Olive husk	24	23.6	48.4
Sunflower shell	48.4	34.6	17
Sugarcane bagasse	45	32	25
Sweet sorghum bagasse	45	27	21
Switchgrass	50	40	20
Wheat straw	40	25	20
Barley straw	45	38	14
Hardwoods	45	30	20
Softwoods	42	27	28
Grasses	40	50	30
Pine	42	21	33
Poplar	44	20	29
Corn stover	38	23	20
Napier grass	47	31	22
Bamboo	45	24	20
Rice Husk	37.1	29.4	24.1
Reed	53.86	10.60	8.76
Candlenut shell	25.77	28.73	36.02
Empty palm fruit bunch	60	22	18
Alfalfa	33	18	8

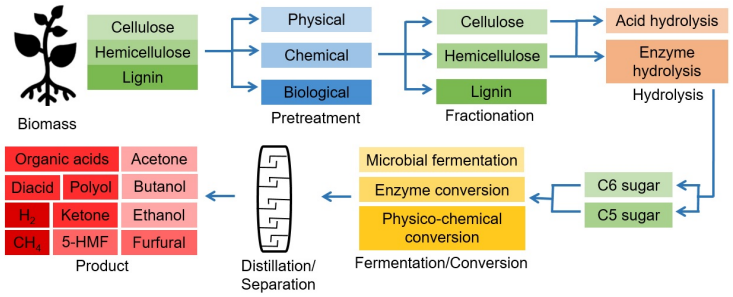

Fig. 1. Process flow diagram for production of ethanol from lignocellulosic biomass

industrial and academic researchers.

2. Lignocellulosic biomass and pretreatment methods

Lignocellulosic biomasss (softwood, hardwood and herbaceous crops) is a plant based non-edible material that is considered abundant and alternative to non-renewable energy source. It includes agricultural/forestry residues, energy crops, garden trimmings, and household garbages. It mainly is composed of three polymer layers such as cellulose, hemicellulose and lignin. The composition of cellulose, hemicellulose and lignin for hardwood and softwood vary as $40-44\%, 15-35\%, 18-25\%$ and $40-44\%, 30-32\%, 25-32\%$, respectively [13]. Cellulose is the homogeneous polysaccharide that is composed of D-glucose linked via $\beta-1-4$ glycosidic linkage. Hemicellulose is a heterogenous polysaccharide composed of pentose (α-L-arabinose and β-D-xylose), hexose (β-D-glucose, β-D-mannose, and α-D-galactose), and uronic acids (α-D-4-O-methylgalacturonic, α-D-glucuronic, and α-D-galacturonic acids) [14]. Lignin is a complex matrix with polyaromatic compounds (phenylpropane units) connected through different linkages. Furthermore, lignin is linked to hemicellulose via ether and carbon-carbon linkages. Lignin layer provides rigidity and protection of polysaccharides towards microbial and animal attack [15].

The fraction of lignin compounds in lignocellulosic biomass are not essential for the ethanol production. However, it is important to breakdown the lignin to increase the exposure of cellulose and hemicellulose to cellulase and hemicellulase enzymes. The lignin and hemicellulose layer are partially or completely solubilized during pretreatment. Pretreatment is an important process to overcome the recal-

citrance caused by the hemicellulose and lignin structure through structural modification. During pretreatment the inter- and intra-hydrogen bond linkages are cleaved causing changes in the structure of lignin, hemicellulose, and cellulose. However, the degree of modification depends upon the type of pretreatment techniques [16, 17]. The major concern over pretreatment method are the generation of sugar degradation compounds and phenolic compounds. Phenolic compounds are composed of various substituted and hydroxyl groups (carboxylic, aliphatic, acyl, and aldehyde) linked to the aromatic structures. These compounds inhibit the activity and functions of enzyme and microbes during saccharification and fermentation process, respectively [18]. Therefore, numerous detoxification process using ion exchange resins, alkali treatment (lime), charcoal, and enzymes are available to neutralize the inhibitors present in the hydrolysate after pretreatment [19].

3. Saccharification and fermentation process

After pretreatment, lignocellulose biomass is proceeded to saccharification and fermentation. Due to the multi-step of the biorefining process, several reactors are required for each step and also the additional separation units are required. This complex process makes the cellulosic ethanol economically unviable. Therefore, various designs of the multi-step process have been developed with state-of-art concept to combine two or more steps in one reactor to save both working time and working space Fig. 2. The process that includes saccharification of biomass (hydrolysis using enzymes to release sugars) followed by fermentation is termed as Separate Hydrolysis and Fermentation (SHF) [20]. On the other hand, merging of the saccharification and fermentation in a single process is termed as Simultaneous Saccharification and Fermentation (SSF). Another process that includes saccharification and fermentation of hydrolysate obtained during saccharification and pretreatment is termed as Simultaneous Saccharification and Co-Fermentation (SSCF) [21]. A single process that is a combination four biological events, such as enzyme production, saccharification, fermentation of C6 sugars, and fermentation of C5 sugars is termed as Consolidated bioprocessing (CBP) [22]. A detail illustration of the SHF, SSF, SSCF, and CBP process has been shown in Fig. 2. The advantage and disadvantage of the individual process has been summarized in Table 2.

3.1. Separate Hydrolysis and Fermentation (SHF)

Separate hydrolysis and fermentation (SHF) is a process by which the saccharification and fermentation process are carried out sequentially. In this process, the conditions for saccharification and fermentation are independent. The requirements of enzyme loadings are less for SHF process than the simultaneous saccharification and fermentation process [23]. The major advantage of SHF is that the two individual process are performed separately and factors (temperature, pH, and stirring) optimized separately (Table 2).

The enzymatic saccharification is usually performed at 50°C, pH 4.5-5.0 during SHF process with less enzyme loading. Cellulose hydrolysis, which uses enzymes in heterogeneous reactions, involves attacks glycosidic linkage between each monomer of cellulose by the activity of endoglucanase and exoglucanase. Oligomers and cellobiose are converted to monomers by catalysis of $\beta - 1,4$ glucosidase [24].

Most of the cellulase enzymes are extracted from decomposer fungi, such as *Trichoderma reesei, Trichoderma viride* and *Fusarium solani* [25]. Enzymes act only on polysaccharides without changing the phenol fraction of lignin structure, resulting in purer fermentable solutions. On the other hand, fermentation process for production of ethanol is performed at temperature 30-35°C, pH 4.0-6.0. The fermentation of sugars in the hydrolysate obtained after enzymatic saccharification is carried out using commonly used yeast strain (*S. cerevisiae*) [26].

However, native strain of *S. cerevisiae* cannot ferment pentose sugars to ethanol. In metabolic pathway, xylose utilization is regulated by xylulokinase through oxidation and reduction reactions and convert to xylitol by a reaction driven by a co-factor, NADPH. Subsequently, xylitol is oxidized to D-xylulose driven by NAD+. Then, D-xylulose enters to Pentose Phosphate Pathway (PPP), and changes to xylulose-5-phosphate. Therefore, the regulation of xylose utilization is governed by NADPH and NAD+ that are involved in aerobic condition [27]. Therefore, genetic engineered *S. cerevisiae* containing pentose metabolism requires optimal feeds of aeration to balance the cofactor pools, and the failure leads to the production of xylitol and CO_2, but not ethanol. Therefore, different wild species or genetic engineered species have potential to ferment pentose and hexose sugar for production of bioethanol [28]. In some studies, engineered species of *S. cerevisiae* have been used for fermentation of hexose and pentose sugars [29]. Genetically modified strains are key microorganisms for creating a bioeconomy for producing bioethanol from lignocellulosic biomass and are a cell factory platform to increase the general value of renewable biomass.

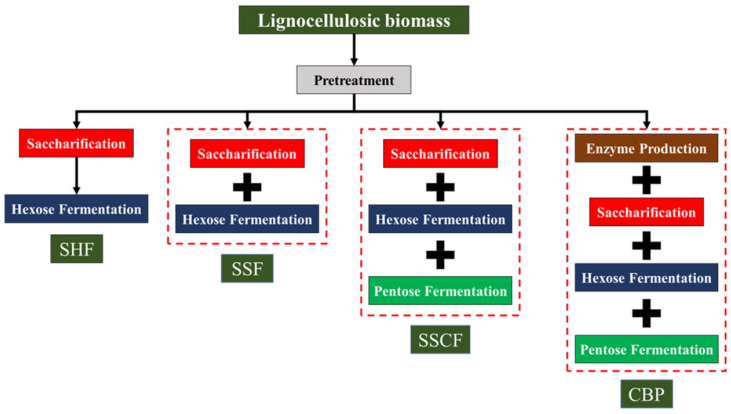

Fig. 2. Different designs of biorefining process for cellulosic ethanol production that combine saccharification and fermentation in one reactor

3.2. Simultaneous Saccharification and Fermentation (SSF)

Simultaneous Saccharification and Fermentation (SSF) is a process that includes liquefaction stage, hydrolysis of biomass to sugars, utilization of sugars by yeast during fermentation for ethanol production. The advantage and disadvantage of the SSF process is summarized in Table 2. During the SSF process, the rate of saccharification is slowed down due to the interference caused by hemicellulose and lignin present in the lignocellulosic biomass [30]. In addition, the crystallinity of cellulose hinders the access of cellulase enzyme during saccharification. One of the main drawbacks of this process is the compromise of optimal conditions to allow the progress of saccharification and fermentation in the same reactor [30].

However, the production cost for the ethanol produced by SSF process is lower than SHF process due to the reduction for the cost of reactor, facility and utility. SSF is usually preferred over SHF due to (a) cost reduction, (b) lower contamination, (c) lower equipment cost, and (d) lower enzyme inhibition, and (e) increase ethanol yield [31]. SSF process has been demonstrated exhibit reduced glucose feedback inhibition because the glucose released from cellulose substrate is continuously consumed by yeast for ethanol production. Rana et al compared the performance of SHF and SSF process for the production of ethanol from wet exploded corn stover (WECS) and loblolly pine (WELP). Higher ethanol yield of 17.3 g/L and 15.4 g/L was reported for WECS and WELP by performing SSF process using commercial enzymes such as Celluclast 1.5 L and Noxozyme 188. The overall yield of ethanol was higher for SSF process compared to SHF process under similar conditions [32]. Dahnum et al signified the importance and effect of SHF and SSF process on incubation period of fermentation process. A maximum ethanol yield of 4.74% and 6.05% was obtained during SHF and SSF process with an incubation period of 72 h and 24 h, respectively, which was 3-fold decrease for working period in SSF [33]. Another study investigated the effect of temperature and compared the performance of SHF and SSF process. The optimum temperature for saccharification and fermentation of SHF process was maintained as 45°C and 37°C, respectively. On the other hand, the optimum temperature for SSF process was maintained at 37°C. Ethanol productivity of 0.837gl^{-1} h^{-1} and 0.313gl^{-1} h^{-1} was obtained for SSF and SHF process with incubation time of 30 h and 96 h, respectively from steam exploded wheat straw [34]. Therefore, it is clearly understood that SSF process has many advantages over SHF process.

3.3. Simultaneous Saccharification and Co-Fermentation (SSCF)

Simutaneous Saccharification and Co-Fermentation (SSCF) process is considered to be a promising method for bioconversion of lignocellulose materials to bioethanol. It overcomes the drawback of SHF process such as high capital cost, longer process duration, and inhibition of enzyme as discussed in previous section. However, there are still some concerns similar to SSF process such as process optimal condition compromise, increased water insoluble solids (WIS) and inhibition caused by ethanol [35]. Furthermore, the lower enzyme load, shorter process duration, utilization of hexose and pentose sugars make this process has advantages over SHF and SSF process. Olofsson et al studied the effect of substrate (wheat straw) and enzyme loading on SSCF process. The fermentation process was performed using a recombinant strain *S*. cerevisiae (TMB3400) having the potential to utilize xylose sugars for ethanol production.

Table 2. The advantage and disadvantage of the SHF, SSF, SSCF, and CBP process

Mode of operation		Characteristics
Separate Hydrolysis and Fermentation (SHF)	Advantage	- Fermentation performed using a clear broth without any solid material - Individual optimum conditions - Possibilities of recycling yeast after fermentation - Facilitates mass transfer without interference - Higher yield of bioethanol - Reduced risk of contamination and inhibition
	Disadvantage	- Inhibition of enzymes caused by monomers, dimers and oligomers - Higher capital cost - Longer process duration - High enzyme load/dosage
Simultaneous Saccharification and Fermentation (SSF)	Advantage	- Low capital cost and investment - Shorter process duration - Lower enzyme load/dosage - Monomers, dimers, and oligomers concentration relatively low due to utilization by microbes leading to lower enzyme inhibition activity - Higher ethanol yield and productivity
	Disadvantage	- Optimal conditions of saccharification anc fermentation process are compromised - Resistance to mass transfer - Inhibition of microbes/enzymes by ethanol - Broth includes insoluble solids inhibiting the microbes/enzyme activity - Reduced possibility in recycle of yeast due to difficulty in separation from solids
Simultaneous Saccharification and Co-Fermentation (SSCF)	Advantage	- Low capital cost and investment - Incorporation and fermentation of both C_6 and C_5 sugars - Shorter process duration - Lower enzyme load/dosage - Monomers, dimers, and oligomers concentration relatively low due to utilization by microbes leading to lower enzyme inhibition activity - Higher ethanol yield and productivity
	Disadvantage	- Optimal conditions of saccharification and fermentation process are compromised - Resistance to mass transfer - Increased inhibitors concentration - Inhibition of microbes/enzymes by ethanol - Broth includes insoluble solids inhibiting the microbes/enzyme activity - Reduced possibility in recycle of yeast due to difficulty in separation from solids - Genetically modified strain are required

This study was concluded by signifying the importance of enzyme and substrate feeding in maintaining lower concentrations of monomers in the medium. Ethanol production of 0.35 g/g of dry biomass was obtained with conversion of xylose increased from 40 to 50% [36].

In another study, the ethanol productivity of 2.6 g/L/h from steam exploded corn stover was obtained with the utilization of glucose (60 g/L) and xylose (10 g/L) by *S.* cerevisiae IPE003. A solid loading of 20%w/v resulted in the conversion of glucan and xxlan by 82% and 82.1%, respectively [37]. Another case investigated the temperature profile and high solid loading to increase the production of ethanol in SSCF process. Two strains of *S.* cerevisiae with the capability of high temperature tolerance and xylose utilization were studied. A study on co-culture in SSCF process showed that the temperature resistant strain was able to maintain the cell viability of the yeast at 42°C in xylose-containing media as the carbon source. Through

Table 2. The advantage and disadvantage of the SHF, SSF, SSCF, and CBP process (continued)

Mode of operation		Characteristics
Consolidated bioprocessing (CBP)	Advantage	- Incorporation of all biological steps in a single process - Low capital cost and investment - Monomers, dimers, and oligomers concentration relatively low due to utilization by microbes leading to lower enzyme inhibition activity - Higher ethanol yield and productivity - Incorporation and fermentation of both C6 and C5 sugars - Shorter process duration - Production of enzyme in single process using engineered strains
	Disadvantage	- Optimal conditions of saccharification and fermentation process are compromised - Resistance to mass transfer - Inhibition of microbes/enzymes by ethanol - Broth includes insoluble solids inhibiting the microbes/enzyme activity - Reduced possibility in recycle of yeast due to difficulty in separation from solids - Genetically modified strain are required - Difficult to engineer and maintain stability of strains

alleviating of ethanol in the media and less sugar accumulation, the ethanol yield of 59.8 g/L was obtained [38]. Therefore, it is clearly understood that SSCF process has merits over SHF and SSF process in accordance to the concept of process intensification.

3.4. Consolidated bioprocessing (CBP)

Consolidated bioprocessing (CBP) is a process that includes four biological processes i.e. enzyme production, saccharification, hexose (glucose, mannose, galactose) fermentation, and pentose (xylose, arabinose) fermentation in a single reactor [22]. CBP offers higher process efficiency and lower cost than processes featuring dedicated cellulase production [39]. Over the past few years, much effort has been put into reducing the cost of producing cellulase enzymes. Still it has been a challenge to run a single reactor with combination of all biological processes with the features for substrate utilization and formation of product. Liu et al performed CBP for the production of bioethanol from lignocellulosic biomass using Clostridium thermocellum DSM 1237. The study evaluated the effect of temperature, carbon sources, and substrate on the performance of the strain during CBP process. An ethanol yield of 0.68 g/L was obtained from alkali pretreated sugarcane bagasse [40]. Another study reported ethanol yield of 62 g/L from mixed lignocellulosic biomass feedstock (high solid loading) during partial CBP process.

The bioethanol conversion was 0.24 g/g and ethanol productivity of 2.82 g L^{-1} h^{-1} was obtained [41]. Vaid et al investigated the performance of one-pot CBP using Saccharum spontaneum biomass (SSB) for bioethanol production. The SSB was subjected to deep eutectic solvent (DES) pretreatment using choline chloride-glycerol and $Ca(OH)_2$.An ethanol yield of 173.61mg/g was obtained from SSB after pretreatment and one pot CBP process. The study provided an insight towards mechanism in the one-pot CBP process and the observations were in agreement by performing different characterization techniques [42]. Chen et al developed a strategy to construct a consortium of yeast strains for expressing lignocellulytic enzymes and producing cellulosic ethanol.

The engineered yeast strains with genes expressing Y5/EG-CBH-BGL (cellulases) and Y5/XxnII-XylA (xylanases) were developed and the CBP process was operated. An ethanol yield of 1.61 g/L at 144 h was achieved from steam exploded corn stover [43]. Therefore, it has been proven that CBP process is a promising approach for ethanol production from different lignocellulosic biomass than SHF, SSF, and SSCF process.

When compared to other biorefining processes, the feasibility of each of these processes depends on a variety of factors, including the feedstock and the available technology [44]. For example, SHF may be more suitable for producing ethanol from simple sugars, such as glucose, while SSCF may be more suitable for producing biofuels from lignocellulosic feedstocks. The reason for this is that

lignocellulosic feedstocks contain multiple types of sugars, including both glucose and xylose, which can be fermented into ethanol. However, most yeast strains used in conventional fermentation processes are not capable of fermenting both glucose and xylose simultaneously [45]. SSCF overcomes this limitation by using specialized microorganisms that are able to ferment both types of sugars. This allows for a higher overall yield of ethanol compared to processes like SHF, which only ferment one type of sugar. Additionally, SSCF can also be more cost-effective compared to other processes because it can utilize low-value lignocellulosic feedstocks that would otherwise go to waste [46]. This makes SSCF a more environmentally friendly and sustainable option compared to other processes. However, it is important to note that SSCF can also be more complex and require specialized microorganisms and equipment, which can increase the cost of production. Additionally, the process may also have lower productivity compared to other processes, such as SHF.

SHF is considered one of the most cost-effective processes for producing ethanol from simple sugars such as glucose. This is because SHF is a relatively simple and inexpensive process that can be performed in a single vessel. The main costs associated with SHF are the costs of the feedstock, the enzyme used to break down the starch or sugar into simpler sugars, and the yeast or bacteria used for fermentation. SSF is considered a cost-effective process for producing ethanol from low-value feedstocks, such as agricultural waste and it can also reduce the cost of production compared to liquid-state fermentation processes [47]. However, SSF can also have lower yields and productivity compared to liquid-state fermentation processes, which can offset the cost savings from using low-value feedstocks. SSCF is often considered a cost-effective process for producing ethanol from lignocellulosic feedstocks, such as agricultural waste and wood chips. This is because SSCF can utilize low-value lignocellulosic feedstocks and increase the overall yield of ethanol compared to other processes, such as SHF. However, SSCF can also be more complex and require specialized microorganisms and equipment, which can increase the cost of production [48]. Additionally, the process may also have lower productivity compared to other processes, such as SHF. The cost-effectiveness of CBP will depend on the specific circumstances and goals of the producer. CBP is a process that aims to optimize the use of the carbon in the feedstock to maximize the overall production of biofuels and other valuable products. CBP can be more complex and require specialized equipment and processes compared to other biorefining processes, which can increase the cost of production. However, CBP can also increase the overall production of biofuels and other valuable products, which can offset the additional cost of the process [49]. Currently, several commercial plants are in operation to produce second-generation or lignocellulosic ethanol by using SHF and SSCF technologies, for example Abengoa Bioenergy (Spain), Green Plains Renewable Energy (USA) and Raízen Energy (Brazil).

4. Ethanol production using recombinant engineered microbes

The catastrophic effects caused by fossil fuel pollutants, such as the global warming and greenhouse effect, are of great concern to global society, coupled with their potential for depletion and high cost. With the ever-increasing demand for cost-effective, environmentally friendly and sustainable energy sources, bioethanol energy is the choice. However, ethanol production from lignocellulosic biomass is faced by many obstacles and challenges. A widely accepted approach to overcome some of these obstacles and challenges is by the strain improvement technology. The strategies for strain improvement based on the current tools and technology are acclimatization, natural screening and selection, direct mutagenesis, or genetic engineering [50].

The yeast strains of *S*. cerevisiae, also known as brewery or baker's yeast, have been studied and developed over the past several decades due to their advantages, such as (a) Generally Recognized as Safe (GRAS) status, (b) Ethanol productivity, (c) sugar conversion, (d) tolerance property, (e) consistency in ethanol production, and (f) genome well studied [51]. S. cerevisiae is currently considered to be one of the model organisms representing eukaryotic members for advanced genetic engineering, molecular biology, genetics, synthetic biotechnology. S. cerevisiae is able to utilize hexose sugar in a catabolic process via glycolysis and produce ethanol and carbon dioxide. However, most of native strains of *S*. cerevisiae cannot utilize C_5 sugars such as L-arabinose and D-xylose for the ethanol production as primary carbon source. In fact, they can ferment pentose when glucose is depleted, which prolong the fermentation time and make the industrial production becomes infeasible. This is due to imbalance of co-factors, NADPH and NAD+, lack of pentose-specific transporter proteins and enzymes [45]. To overcome this limitation, several features have been applied for strain improvement, such as expressing genes, D-xylose isomerase, involved in conversion of pentose to metabolic intermediates, D-xylulose, that can be catabolized in the pentose phosphate pathway (PPP) (or hexose monophosphate shunt and the HMP shunt). Then, D-xylulose is phosphorylated to xylulose-5-phosphate by the function of xylulokinase and this xylose-derived inter-

mediate can enter to the non-oxidative phase of PPP and subsequently to glycolysis [52].

Lignocellulose biomass contains cellulose $35-50\%$ and hemicellulose $25-40\%$, therefore utilization of both cellulose and hemicellulose in the forms of hexose and pentose will maximize the values of biomass and it is a solution for economic viability [2]. With respect to this ideal concept, various strains of microorganisms (yeast, actinomycetes and bacteria) have been investigated in the process with the expected phenotypes to increase ethanol productivity and to co-ferment glucose and xylose [45]. However, wild strains exhibit several limitations, such as low sugar conversion rate and low utilization of substrate. In general, the microbial strains selected for industrial application should meet various criteria, including culture growth rate, substrate utilization for ethanol production, tolerance towards environmental stress and ethanol concentration, temperature and pH tolerance, tolerance for osmotic pressure, and tolerance for toxic/inhibitory compounds [51]. However, a single wild strain of yeast, bacteria, or actinomycetes does not exhibit most of these properties. Therefore, to achieve these characteristic for the developed strain, different genetic engineering strategies have been adopted by overexpressing native genes/pathways regulating the desired phenotypes in the host strain or deleting native genes/pathways competing the desired metabolic pathway Fig. 3. The targeted phenotypes for recombinant strains that relate to promotion of cellulosic ethanol are enhanced substrate utilization, cofermentation of C_5 and C_6 sugars, improved tolerance to ethanol and harsh condition, augmented cellular secretion and transportation pathway, and creating the cell-surface motifs/modules of multi-enzymes with hydrolysis activities [20].

A widely utilized technique to modify the phenotype of the targeted wild strain (host strain) is genome editing [53] (Fig. 3). Genome editing allows multiple and simultaneous modification of genes in host strain. Promoter engineering is another method that includes identification of gene of interest for the expression of gene responsible for the production of enzymes to utilize hexose and pentose sugar, and tolerance towards inhibitor compounds. Metabolic engineering is a strategy to improve the pathway for enzymes production, such as xylose reductase and xylitol dehydrogenase for the utilization of the xylose during fermentation [54]. Randomized mutagenesis is a strategy to mutate strain by UV exposure and/or chemicals. Through this method, the strain develops tolerance for ethanol, temperature and pH resulting in higher yield of ethanol. Sequencing of whole genome (gene shuffling) is also a different strategy to deal with traits of complex polygenic phenotypes. The genes that are responsible for the tolerance of ethanol, temperature, and increased ethanol productivity are pair with the genome of species. Another approach is through evolutionary engineering that involves improvement of the culture growth, utilization of xylose, and fermentation kinetics through batch or chemostat culture method. Production of enzymes, such as xylose reductase, xylitol dehydrogenase, utilization of pentose and hexose sugars through rational protein design is another strategy [55]. Another method involves the identification of alleles and phenotypic traits in strains exhibiting tolerance towards high temperature and high ethanol concentration [56]. Currently, there are many microorganisms that are selected as host strain for genetic engineering for the purpose to be applied for cellulosic ethanol production (Fig. 3). A select list of genetically modified strains developed over the past two decades for the production of lignocellulosic bioethanol is given in Table 3.

The examples of yeasts for this purpose are *S.cerevisiae, Kluyveromyces marxianus, Pichia pastori, Scheffersomyces stipites*. In addition to yeast, several of bacterial species are selected for this goal as prokaryote members such as *Escherichia coli, Zymomonas mobilis*, and *cyanobacteria*. Also, thermophilic bacteria are also selected due to their tolerance to stress condition, especially when they are applied in SSF, SSCF and CBP process due to increased compatibility to pretreatment condition, and examples of these members are *Geobacillus, Thermoanaerobacter, Clostridium* [4].

One example to promote production of lignocellulosic ethanol, the native genes XYL1 and XYL2 of Scheffersomoces stipitis (formerly known as Pichia stipites) was introduced to a wild strain of *S.* cerevisiae results in the expression of xylose reductase ad xylose dehydrogenase enzymes. Introduction of these genes through genome editing technique resulted in the assimilation of the xylose by the wild strain *S.* cerevisiae [75]. Additionally, XYL3 of *S.* stipites was transformed into *S.* cerevisiae for the production of xylulokinase enzyme resulted in improved xylose utilization [76]. Based on the concept, the ethanol production should be improved with utilization of both hexose and pentose sugars during fermentation process by using engineered strains. However, the ethanol productivity and yield were reported to be lower than that obtained with the wild strain S. stipites and traditional yeast strains. Combined fermentation of glucose and xylose results in the utilization of glucose as the primary carbon source for production of ethanol. After glucose concentration was depleted, this recombinant *S.* cerevisiae utilized remaining xylose for ethanol fermentation [45]. This observation suggested that the success of ethanol production is not

Table 3. List of genetically modified microorganisms for efficient lignocellulosic ethanol production.

Wild type	Recombinant strain	Modification	Substrate	Remarks	Reference
		Bacillus subtilis			
B.subtilis WB600	NS:Z	Insertion of alcohol dehydrogenase genes from Z. mobilis (adhZ) / S. cerevisiae (adhS) and pyruvate decarboxylase gene (pdcz) from Z. mobilis followed by fusion of genes	Potatoes	Ethanol production in 96 h fermentation - 21.5 g/L	[57]
		Clostridium cellulolyticum			
Clostridium cellulolyticum ATCC 35319	CC-pMG8	Clostridium cellulolyticum carrying plasmid pMG8; macrolide-lincosamide-streptogramin resistance	Cellulose	decreased pyruvic acid 60% increased ethanol production by 53% and acetate production by 90%	[58]
		Clostridium thermocellum			
C. thermocellum	LL1319	adhE, nfnA, nfnB and adhA genes from Thermoanaerobacterium saccharolyticum introduced & hydrogen producing genes deleted	solubilized Avicel	ethanol yield – 48% theoretical titer – 15.02 g/L max. sp productivity – 0.64 g/L ethanol/ h	[59]
C. thermocellum	LL1381	Deletion of hydG and ech from LL1319	solubilized Avicel	ethanol yield – 39% theoretical titer – 12.9 g/L max. specific productivity – 0.55	[59]
C. thermocellum	AG553	Deletion of side products (acetate, lactate, H_2, formate) pathways	model crystalline cellulose Avicel	63.5% of the theoretical ethanol yield; 3 fold increase compared to the wild type	[60]
C. thermocellum DSM1313 (Ethanol tolerant mutant)		Deletion lactate dehydrogenase genes	Minimal medium	30 higher ethanol production compared to wild strain	[61]
C. thermocellum DSM1313	CTH-*pdc*, CTH-*adh*, CTH-*pdc-adh*	inserted pyruvate carboxylase (*pdc*) and alcohol dehydrogenase (*adh*) genes from	CTFUD medium	CTH-*pdc* strain increased ethanol production by 2 fold; adh impaired the growth	[61]
		E. coli			
	K011	Inserted pyruvate decarboxylase and alcohol dehydrogenase genes from Z. mobilis; suppressed succinate production	Acid hydrolysed corn fiber and corn germ meal	ethanol concentration 3.47 & 3.42%; carbon recovery 92 & 98%	[62]
	SL40	fosfomycin resistant mutant of E. coli K011	Acid hydrolysed corn fiber and corn germ meal	ethanol concentration 3.17 & 2.92%; carbon recovery 94 & 100%	[62]

Table 3. List of genetically modified microorganisms for efficient lignocellulosic ethanol production (continued).

Wild type	Recombinant strain	Modification	Substrate	Remarks	Reference
		Kluyveromyces marxianus			
Kluyveromyces marxianus		display endoglucanase from Trichoderma reesei and β-glucosidase from Aspergillus aculeatus on the cell surface	β-glucan	Direct conversion of β-glucan to ethanol possible; 92.2% theoretical ethanol yield	[63]
		Pichia pastoris			
Pichia pastoris		display of endoglucanase (EG), exoglucanase (CBH) and β-glucosidase (BGL) on the cell surface	carboxymethyl cellulose (CMC)	Direction conversion of CMC to ethanol achieved; maxmum ethanol concentration 5.1 g/L	[64]
		Saccharomyces cerevisiae			
S.cerevisiae MM476 (SF7)	SF7-Ft3	*XYL*1 and *XYL*2 genes from Candida tropicalis and *XKS1* gene from *S. cerevisiae MM476*	wheat, maize, Miscanthus	xylose consumption & ethanol yield: engineered strain - 95% & 2.08 g/L; native strain - 13% and 0.56 g/L	[65]
S.cerevisiae Ethanol Red IM ***(ER)***	ER T12	*Talaromyces emersonii* glucoamylase encoding gene tem G_0Qpt and native *T*. emersonii α-amylase encoding gene (temA) were expressed	Starch	synthesis ethanol from starch in single step; ethanol concentration 89.35 g/L; carbon conversion – 87%; 90% reduction in endogeneous enzyme	[66]
S.cerevisiae M2n	M2n T1	Talaromxces emersonii glucoamylase encoding gene tem G_Qpt and native *T. emersonii* α-amylase encoding gene (temA) were expressed	Starch	synthesis ethanol from starch in single step; ethanol concentration 98.13 g/L; carbon conversion – 94%; 90% reduction in endogeneous enzyme	[66]
S. cerevisiae	SXA-R2P-E	engineered to express xylose isomerase	glucose and xylose	0.44 g ethanol/g of sugars (glucose and xylose); 0.43 – 0.46 g ethanol/g of sugars consumed in lignocellulose hydrolysate; low xylose consumption rate	[67]

Table 3. List of genetically modified microorganisms for efficient lignocellulosic ethanol production (continued).

Wild type	Recombinant strain	Modification	Substrate	Remarks	Reference
		Saccharomyces cerevisiae			
S.cerevisiae CCUG53310		Over expression of PRS3, RPB4 and ZWF1 genes	Eucalyptus globulus wood and corn cob hydrolysates	PRS3 enhanced fermentation rate and productivity upto 32% and 48%, respectively; ZWF1 and RPB4 improved inhibitor tolerance	[68]
S.cerevisiae PE-2		Over expression of PRS3, RPB4 and ZWF1 genes	Eucalyptus globulus wood and corn cob hydrolysates	PRS3 enhanced fermentation rate and productivity upto 32% and 48%, respectively; ZWF1 and RPB4 improved inhibitor tolerance	[68]
S.cerevisiae		Over expression of genes RCK1	Glucose / xylose	Improved assimilation of glucose and xylose, higher tolerance to acetic acid (40% lower reactive oxygen species), double the ethanol productivity compared to parent strain	[69]
S.cerevisiae PE-2	PE-HAA1, PE-PRS3, PE-HAA1/PRS3	PE-2 △GRE3, pMEC9001 PE-2 △GRE3, pMEC9002 PE-2 △GRE3, pMEC9003	Paulownia tomentosa hydrolysate	Growth is favoured in glucose; higher tolerant to acetic acid stress; HAA1 favoured both glucose and xylose consumption; PSR3 favoured glucose consumption but did not affect xylose consumption	[70]
S.cerevisiae		Down-regulation of SSK2, PPG1, and PAM1		Improved ethanol and heat tolerance	[71]

guaranteed, although the necessary metabolic pathways or enzymes for xylose utilization (e.g. xylose isomerase *xzlA*, xylulokinase *xylB*) and pentose metabolism pathway (transketolase *t[ktA* o transaldolase talB)) [77] are existed in the working strains. The other parameters of culturing should also be controlled, such as aeration rates to control the balance of redox status to drive the metabolisms to completely consume both type of sugars.

One of the current strategy that has been developed from genetic engineering and metabolic engineering techniques to develop various recombinant yeast/bacterial strains with capability to produce cellulase enzymes and enable the CBP operation for lignocellulosic ethanol production [78] (Fig. 4). A single or sets of genes encoded for cellulase enzymes (exoglucanase endoglucanase, β-glucosidase) or accessory proteins (Carbohydrate Binding Modules (CBM), anchor protein, scaffolding protein) are introduced into the wild type *S*. cerevisiae in the forms of

Table 3. List of genetically modified microorganisms for efficient lignocellulosic ethanol production (continued).

Wild type	Recombinant strain	Modification	Substrate	Remarks	Reference
		Zymomonas mobilis			
Zymomonas mobilis		Insertion of genes from *E.coli* encoding L-arabinose isomerase (*acaA*), L-ribulokinase (araB), L-ribulose-5-phosphate-4-epimerase (araD), transaldolase (talB), and transketolase (*tktA*)	arabinose	98% of maximum theoretical yield of ethanol from arabinose (25 g/L) as a sole carbon source; low arabinose consumption rate	[72]
Zymomonas mobilis		Insertion of *Escherichia coli* genes encoding xylose isomerase (xylA), xylulose kinase (xylB), transketolase (tktA), and transaldolase (talB))	Xylose	Simultaneously fermented glucose and xylose; ethanol yield 86%	[73]
Zymomonas mobilis ATCC 31821 ZM4	Z.mobilis ZM4 (pZB5)	Contains xylose isomerase, xylulokinase, transketolase, and transaldolase genes from *E. Coli*	Glucose and Xylose	Ethanol yield 0.46 g/g when glucose and xylose were fed at equal concentrations of 65 g/L. Yield decreased with higher sugar concentrations due to product inhibition	[74]

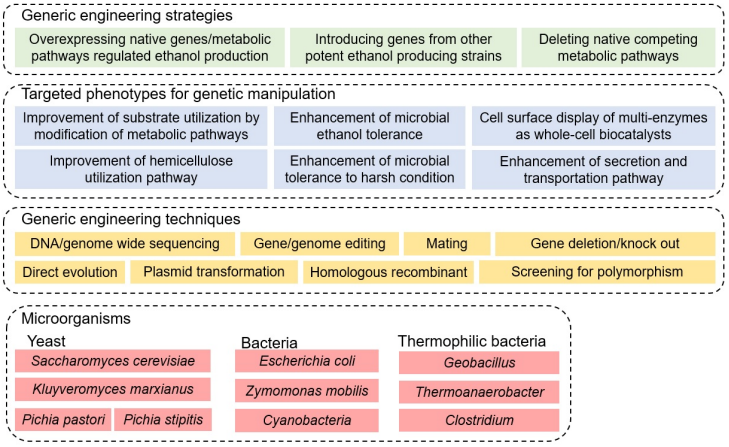

Fig. 3. Different designs of biorefining process for cellulosic ethanol production that combine saccharification and fermentation in one reactor

espisomal expression (via plasmid) or chromosomal integration [79]. The targeted exogenous gene/gene cassette are regulated by operational modules, which is composed of promoter (to response to transcriptional factor (TF)), signal peptide (to determine the protein/enzyme destination, modification, secretion and transportation), and terminator (to determine the stop point of transcription). After the cellulase enzymes and accessory proteins are synthesized, they are post-translational processed and translocated to endoplasmic reticulum (ER) and golgi body for protein

secretion to extracellular compartments. Once all cellulases and accessory proteins assemble together to form tethered cellulases or complex cellulases (so-called cellulosome), the recombinant yeast/bacteria could adhere to the lignocellulosic fibrils and hydrolyze the substrates to hexose and pentose sugars [80]. The recombinant strains could also additionally be introduced with the genes encoded for sugar transporters to promote the uptake rate of the sugar to improve the yield of ethanol. Furthermore, the genes responsible for stress tolerance phenotypes, such as ethanol stress, or ROS stress are transformed to these recombinant microbes to improve survival rate in inappropriate condition, such as high salt concentration or extreme pH Additionally, the glucose inhibition feedback pathway could also be deleted or knockdown to allow host strain to uptake high substrate concentration and enhance ethanol production [81]. With this scheme of recombinant strategy, the genetic engineered microbial strain has characteristic to function in saccharification and fermentation that fits well in the SSF, SSCF and CBP processes.

5. Case studies in the development of recombinant yeast for lignocellulosic ethanol production

S. cerevisiae is a widely popular industrial host strains and many engineering efforts have gone into creating innovative yeast cell strains to produce ethanol and a biochemical product [82]. Due to their properties, including (a) tolerance to high sugar concentrations, low pH and ethanol, (b) ability to produce high titer of ethanol, (c) resistance to inhibitors, and (d) safety, this make yeast an ideal candidate [20]. In addition, the complete known genome sequence of yeast strains allows them to be easily manipulated using advanced tools in synthetic biology and genetic engineering [83]. For instance, Cunha et al developed cell surface engineered yeast for production of ethanol from corn cob and cheese whey through CBP by using epigenomic gene transformation [29]. The study investigated the performance of engineered yeast strains exhibiting stress resistance, thermotolerance and cell surface display of cellulolytic enzymes. The increased ethanol yield for 2.5 -fold from multi feedstock was observed (lignocellulose biomass and diary by-products), thereby contributing to the economic viability of the ethanol process and its establishment [84].

Similar concept of development was done to genetically engineer various yeast strains for optimal secretion ratio of cellulase enzymes (β-glucosidase (BGLI), endoglucanase (EGII) and cellobiohydrolase (CBHI)) in the forms of freely secreted cellulases. It was found that 56% of cellulose in substrate were converted to glucose, and 4 g/L of ethanol yield was obtained [85]. Based on the same idea, the engineering Pichia pastoris with expression and in vitro assembly of cellulosome modules (containing endoglucanase from C. thermocellume. exoglucanase from Yarrowid lipolvtica β-glucosidase from Thermoanaerobacterium thermosaccharolvticum and a CBM from Thermobifida fusca) was constructed. This recombinant yeast was tested to convert the carboxymethyl cellulose (CMC) to ethanol with the yield of 5 g/1 and it also showed higher cell density during fermentation compared to *S*. cerevisiae [63]. Another thermotolerant yeast strain, Kluxueromuces, marxianus, was genetically modified to express the surface-displayed endoglucanase and β-glucosidase originated from Trichoderma resses and Aspergillus aculeatus, respectively to be recombinant yeast that can function in high temperature condition that is optimal for cellulase activities. This strain of *K*. marxianus was proved to convert beta-glucan to ethanol with the conversion rate of 42.4% within 12 h, and equivalent to 92% of the theoretical yield [64].

At present, there are more studies to develop the novel recombinant yeast strains for production of lignocellulosic ethanol in lab scale, nevertheless the technological translation and transfer of this lab-scale success is not readily for industrial scale production. Although there are many success cases to express exogenous enzymes/proteins involved in saccharification and utilization of sugar for ethanol production. The levels of functional enzymes during saccharification and fermentation are mostly not in satisfy levels due to the expression loads of these components in the host cells, leading to the biological stresses and at the end low ethanol yield [20]. To compromise the expression of these exogenous enzymes/proteins and allow optimal growth/viability of host cells, several approaches could be implemented. For example, the operon of target genes/gene cassettes could be regulated by modification and optimization of regulatory domains, such as promoters, attenuators, transcriptional factors, signal peptides to allow the optimal expression of cellulases. On the other hand, the secretory pathway of these exogenous proteins/enzymes is in needed to deliver these components to the specific action sites, for example, cellulase in extracellular compartment, or sugar transporter in intercellular membrane.

6. Techno-economical concerns for commercial process of lignocellulosic ethanol

Based on the design of biorefining process for production of lignocellulosic ethanol, the final step is the distillation process to recover ethanol from fermentation broth. However, to make the economical feasible processing of distillation,

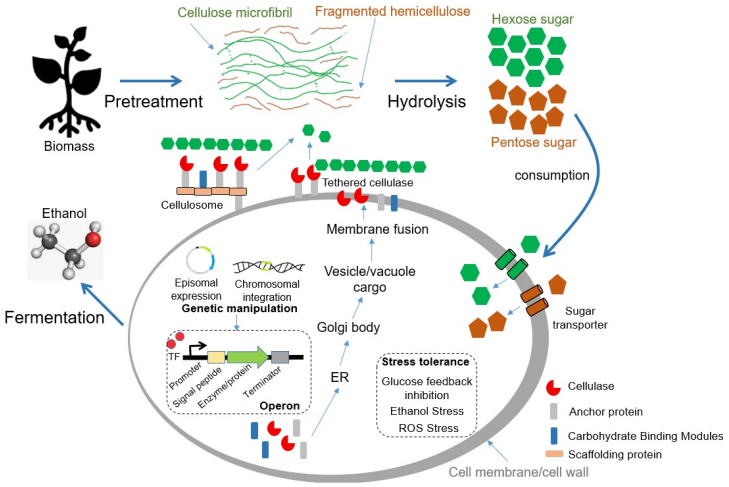

Fig. 4. Characteristics of genetic engineered microorganisms with functions in saccharification and utilization of hexose and pentose sugars for ethanol production

at least 4% of ethanol concentration should be obtained in fermentation broth to compensate to the electricity or heat source cost to supply the distillation tower [85, 86]. According to the mole conversion ratio between glucose (hexose) to ethanol, this means at least 8% of sugars should be available in starting lignocellulose hydrolysate. Due to the proportion of cellulose in lignocellulose biomass is $35-50\%$, the lignocellulose loading ratio in hydrolysis reaction should be higher than 20%, under the assumption that saccharification efficiency almost reach to 100%. However, most of experiments in lab scale research and pilot scale production use less than 20% load of lignocellulose biomass, and mostly $10-15\%$ [87]. This is due to the limitation in mass transfer of lignocellulose in hydrolysis buffer containing cellulase enzymes. Also, most lignocellulose biomass has high inbibition pressure that absorb surrounding water in the reactor very well, making less water concentration in the reaction and less hydrolysis activity ultimately. In this regard, in addition to use of genetic engineered strains with high cellulase activities and high conversion efficiency for ethanol, the state-of-art in process design, instrumentation, operation and optimization should be achieved. The high loading ratio of biomass should be conducted by using the appropriate propeller/mixer to promote the mass transfer rate [88]. The fed-batch biomass loading is also alternative solution to allow the delayed addition of biomass because the early added biomass is already hydrolyzed and more space is available for further loading of substrate [89].

Furthermore, the whole process of biorefining process for lignocellulosic ethanol production should be designed at one time to allow the compatibility of each step and fit well with the phenotypes of the genetic engineered strain. For example, when lignocellulose biomass is pretreated with ionic liquid, a green solvent, that has been demonstrated to be one of highly effective method to allow disintegration of cellulose fibers, modification of crystalline structure of cellulose and removal of lignin, with minimum generation of other inhibitory by-products. However, before proceed to the hydrolysis in SHF or SSF modes, the pretreated biomass should be intensively washed to remove ionic liquid residues. Several studies demonstrated the inhibition effect of ionic liquid on cellulase activities, microbial viability and fermentation efficiency [90]. Therefore, large amounts of wastewater are generated, which increase the cost of the process for wastewater treatment. For this scenario, the genetic engineered microbial strains with ionic liquid tolerance could be the solution and it is proved previously that the ionic liquid tolerant-cellulase producing bacteria is effective biocatalyst in one-pot process for lignocellulosic ethanol production [91]. Another scenario, when the lignocellulose biomass is pretreated with high temperature condition, such as stream explosion, genetic engineered thermophilic bacteria could be a potential option by introducing the genes/pathways to promote ethanol production into the wild type thermophilic bacteria. Last but not least, the related law regulation and society perception should also aware before the design of the biorefining process. For example, the genetic engineered organisms (GMOs) are banned from many countries, and some countries enforce the regulation for GMOs facility with high level of biosecurity, which require high cost investment in facility, operation and maintenance [92]. Insufficient knowledge and awareness of these concerns could lead to infeasibility of the commercial process of

lignocellulose ethanol production.

7. Conclusion

Nowadays, bioethanol is one of the most important sustainable energy to replace fossil fuels with the merits to secure the supply of gasoline and reduce the emission of greenhouse gas. Due to the complexity of biorefining process of ethanol production from lignocellulose, the costs of investment and operation become the main bottleneck and result in the uncompetitive price of lignocellulosic ethanol compared to first generation ethanol. Therefore, the new strategy using genetic engineered microbes with characteristics to promote the saccharification and fermentation is in need. This review discussed the perspectives of development of recombinant microbial strains and compatibility with the process operations. Several technological concerns and economical aspects were also discussed about the current work in the lab scale research and knowledge transfer for industrial production to construct and operate the viable process for global market of lignocellulosic ethanol.

Acknowledgements

The authors are thankful to the King Mongkut's University of Technology North Bangkok (Grant Contract No. KMUTNB-66-BASIC-16) for the financial support during this work.

References

[1] T. Hasunuma and A. Kondo, (2012) *"Consolidated bioprocessing and simultaneous saccharification and fermentation of lignocellulose to ethanol with thermotolerant yeast strains"* **Process Biochemistry *47***(9): 1287–1294. DOI: 10.1016/j.procbio.2012.05.004.

[2] Y.-S. Cheng, P. Mutrakulcharoen, S. Chuetor, K. Cheenkachorn, P. Tantayotai, E. J. Panakkal, and M. Sriariyanun, (2020) *"Recent situation and progress in biorefining process of lignocellulosic biomass: Toward green economy"* **Applied Science and Engineering Progress *13***(4): 299–311. DOI: 10.14416/j.asep.2020.08.002.

[3] M. P. Gundupalli and M. Sriariyanun, (2022) *"Recent trends and updates for chemical pretreatment of lignocellulosic biomass"* **Appl. Sci. Eng. Prog *16***: 5842. DOI: 10.14416/j.asep.2022.03.002.

[4] T. Raj, K. Chandrasekhar, A. N. Kumar, J. R. Banu, J.-J. Yoon, S. K. Bhatia, Y.-H. Yang, S. Varjani, and S.-H. Kim, (2022) *"Recent advances in commercial biorefineries for lignocellulosic ethanol production: Current status, challenges and future perspectives"* **Bioresource technology *344***: 126292. DOI: 10.1016/j.biortech.2021.126292.

[5] J. Qiao, H. Cui, M. Wang, X. Fu, X. Wang, X. Li, and H. Huang, (2022) *"Integrated biorefinery approaches for the industrialization of cellulosic ethanol fuel"* **Bioresource Technology**: 127516. DOI: 10.1016/j.biortech.2022.127516.

[6] L. G. Nair, K. Agrawal, and P. Verma, (2022) *"An insight into the principles of lignocellulosic biomass-based zero-waste biorefineries: a green leap towards imperishable energy-based future"* **Biotechnology and Genetic Engineering Reviews**: 1–51. DOI: 10.1080/02648725.2022.2082223.

[7] A. M. Borrero-Lopez, C. Valencia, and J. Franco, (2022) *"Lignocellulosic materials for the production of biofuels, biochemicals and biomaterials and applications of lignocellulose-based polyurethanes: a review"* **Polymers *14***(5): 881. DOI: 10.3390/polym14050881.

[8] H. Guo, Y. Zhao, J.-S. Chang, and D.-J. Lee, (2022) *"Inhibitor formation and detoxification during lignocellulose biorefinery: A review"* **Bioresource Technology**: 127666. DOI: 10.1016/j.biortech.2022.127666.

[9] B. A. Brandt, T. Jansen, J. F. Görgens, and W. H. van Zyl, (2019) *"Overcoming lignocellulose-derived microbial inhibitors: advancing the Saccharomyces cerevisiae resistance toolbox"* **Biofuels, Bioproducts and Biorefining *13***(6): 1520–1536. DOI: 10.1002/bbb.2042.

[10] S.-J. Ha, J. M. Galazka, S. Rin Kim, J.-H. Choi, X. Yang, J.-H. Seo, N. Louise Glass, J. H. Cate, and Y.-S. Jin, (2011) *"Engineered Saccharomyces cerevisiae capable of simultaneous cellobiose and xylose fermentation"* **Proceedings of the National Academy of Sciences *108***(2): 504–509. DOI: 10.1073/pnas.1010456108.

[11] T. W. Jeffries and Y.-S. Jin, (2000) *"Ethanol and thermotolerance in the bioconversion of xylose by yeasts"*: DOI: DOI:10.1016/S0065-2164(00)47006-1.

[12] A. Duque, C. Álvarez, P. Doménech, P. Manzanares, and A. D. Moreno, (2021) *"Advanced bioethanol production: From novel raw materials to integrated biorefineries"* **Processes *9***(2): 206.

[13] Z. Ahmad, W. W. Al Dajani, M. Paleologou, and C. Xu, (2020) *"Sustainable process for the depolymerization/oxidation of softwood and hardwood kraft lignins using hydrogen peroxide under ambient conditions"* **Molecules *25***(10): 2329. DOI: 10 . 3390 / molecules25102329.

[14] J.-M. Lavoie, R. Beauchet, V. Berberi, and M. Chornet. "Biorefining lignocellulosic biomass via the feedstock impregnation rapid and sequential steam treatment". In: *Biofuel's Engineering Process Technology*. IntechOpen, 2011. DOI: 10.5772/18186.

[15] D. Jose, C. Muenmuang, N. Kitiborwornkul, P. Yasurin, S. Asavasanti, P. Tantayotai, and M. Sriariyanun, (2022) *"Effect of surfactants and Co-surfactants in formulation of noni fruit extract in virgin coconut oil-based emulsion"* **Journal of the Indian Chemical Society *99***(10): 100729.

[16] L. Zhao, Z.-F. Sun, C.-C. Zhang, J. Nan, N.-Q. Ren, D.-J. Lee, and C. Chen, (2022) *"Advances in pretreatment of lignocellulosic biomass for bioenergy production: Challenges and perspectives"* **Bioresource Technology *343***: 126123. DOI: 10.1016/j.biortech.2021.126123.

[17] Y.-S. Cheng, Z.-Y. Wu, and M. Sriariyanun, (2019) *"Evaluation of Macaranga tanarius as a biomass feedstock for fermentable sugars production"* **Bioresource technology *294***: 122195. DOI: 10.1016/j.biortech.2019.122195.

[18] S. Sharma, E. Varghese, A. Arora, K. Singh, S. Singh, L. Nain, and D. Paul, (2018) *"Augmenting pentose utilization and ethanol production of native Saccharomyces cerevisiae LN using medium engineering and response surface methodology"* **Frontiers in Bioengineering and Biotechnology *6***: 132.

[19] E. J. Panakkal, M. Sriariyanun, J. Ratanapoompinyo, P. Yasurin, K. Cheenkachorn, W. Rodiahwati, and P. Tantayotai, (2022) *"Influence of sulfuric acid pretreatment and inhibitor of sugarcane bagasse on the production of fermentable sugar and ethanol"* **Applied Science and Engineering Progress *15***(1): DOI: 10.14416/j.asep.2021.07.006.

[20] J. Sharma, V. Kumar, R. Prasad, and N. A. Gaur, (2022) *"Engineering of Saccharomyces cerevisiae as a consolidated bioprocessing host to produce cellulosic ethanol: Recent advancements and current challenges"* **Biotechnology Advances**: 107925. DOI: 10.1016/j.biotechadv.2022.107925.

[21] T. H. Kim, R. Gupta, and Y. Lee, (2009) *"Pretreatment of biomass by aqueous ammonia for bioethanol production"* **Biofuels: methods and protocols**: 79–91. DOI: 10.1007/978-1-60761-214-8_6.

[22] W. H. Van Zyl, L. R. Lynd, R. den Haan, and J. E. McBride, (2007) *"Consolidated bioprocessing for bioethanol production using Saccharomyces cerevisiae"* **Biofuels**: 205–235. DOI: 10.1007/10_2007_061.

[23] H. Zabed, J. Sahu, A. N. Boyce, and G. Faruq, (2016) *"Fuel ethanol production from lignocellulosic biomass: an overview on feedstocks and technological approaches"* **Renewable and sustainable energy reviews *66***: 751–774. DOI: 10.1016/j.rser.2016.08.038.

[24] L. Peng and Y. Chen, (2011) *"Conversion of paper sludge to ethanol by separate hydrolysis and fermentation (SHF) using Saccharomyces cerevisiae"* **Biomass and Bioenergy *35***(4): 1600–1606. DOI: 10.1016/j.biombioe.2011.01.059.

[25] M. P. Coughlan, (1985) *"The properties of fungal and bacterial cellulases with comment on their production and application"* **Biotechnology and genetic engineering reviews *3***(1): 39–110. DOI: 10.1080/02648725.1985.10647809.

[26] M. Parapouli, A. Vasileiadis, A.-S. Afendra, and E. Hatziloukas, (2020) *"Saccharomyces cerevisiae and its industrial applications"* **AIMS microbiology *6***(1): 1. DOI: 10.3934/microbiol.2020001.

[27] M. A. Tesfay, X. Win, H. Lin, Y. Liu, C. Li, J. Lin, and J. Lin, (2021) *"Efficient L-xylulose production using whole-cell biocatalyst with NAD+ regeneration system through co-expression of xylitol dehydrogenase and NADH oxidase in Escherichia coli"* **Biochemical Engineering Journal *175***: 108137.

[28] N. K. Sharma, S. Behera, R. Arora, S. Kumar, and R. K. Sani, (2018) *"Xylose transport in yeast for lignocellulosic ethanol production: current status"* **Journal of bioscience and bioengineering *125***(3): 259–267. DOI: 10.1016/j.jbiosc.2017.10.006.

[29] J. T. Cunha, P. O. Soares, S. L. Baptista, C. E. Costa, and L. Domingues, (2020) *"Engineered Saccharomyces cerevisiae for lignocellulosic valorization: a review and perspectives on bioethanol production"* **Bioengineered *11***(1): 883–903. DOI: 10.1080/21655979.2020.1801178.

[30] M. Toor, S. S. Kumar, S. K. Malyan, N. R. Bishnoi, T. Mathimani, K. Rajendran, and A. Pugazhendhi, (2020) *"An overview on bioethanol production from lignocellulosic feedstocks"* **Chemosphere *242***: 125080. DOI: 10.1016/j.chemosphere.2019.125080.

[31] J. M. Fox, S. E. Levine, H. W. Blanch, and D. S. Clark, (2012) *"An evaluation of cellulose saccharification and fermentation with an engineered Saccharomyces cerevisiae capable of cellobiose and xylose utilization"* **Biotechnology Journal 7**(3): 361–373. DOI: 10.1002/biot.201100209.

[32] V. Rana, A. D. Eckard, and B. K. Ahring, (2014) *"Comparison of SHF and SSF of wet exploded corn stover and loblolly pine using in-house enzymes produced from T. reesei RUT C30 and A. saccharolyticus"* **SpringerPlus *3***: 1–13. DOI: 10.1186/2193-1801-3-516.

[33] D. Dahnum, S. O. Tasum, E. Triwahyuni, M. Nurdin, and H. Abimanyu, (2015) *"Comparison of SHF and SSF processes using enzyme and dry yeast for optimization of bioethanol production from empty fruit bunch"* **Energy Procedia *68***: 107–116. DOI: 10.1016/j.egypro.2015.03.238.

[34] F. Alfani, A. Gallifuoco, A. Saporosi, A. Spera, and M. Cantarella, (2000) *"Comparison of SHF and SSF processes for the bioconversion of steam-exploded wheat straw"* **Journal of Industrial Microbiology and Biotechnology *25***(4): 184–192. DOI: 10.1038/sj.jim.7000054.

[35] H. Kawaguchi, T. Hasunuma, C. Ogino, and A. Kondo, (2016) *"Bioprocessing of bio-based chemicals produced from lignocellulosic feedstocks"* **Current opinion in biotechnology *42***: 30–39. DOI: 10.1016/j.copbio.2016.02.031.

[36] K. Olofsson, B. Palmqvist, and G. Lidén, (2010) *"Improving simultaneous saccharification and co-fermentation of pretreated wheat straw using both enzyme and substrate feeding"* **Biotechnology for biofuels *3***: 1–9. DOI: 10.1186/1754-6834-3-17.

[37] Z.-H. Liu and H.-Z. Chen, (2016) *"Simultaneous saccharification and co-fermentation for improving the xylose utilization of steam exploded corn stover at high solid loading"* **Bioresource technology *201***: 15–26. DOI: 10.1016/j.biortech.2015.11.023.

[38] J.-Q. Zhu, Q.-J. Zong, W.-C. Li, M.-Z. Chai, T. Xu, H. Liu, H. Fan, B.-Z. Li, and Y.-J. Yuan, (2020) *"Temperature profiled simultaneous saccharification and co-fermentation of corn stover increases ethanol production at high solid loading"* **Energy Conversion and Management *205***: 112344.

[39] L. R. Lynd, W. H. Van Zyl, J. E. McBride, and M. Laser, (2005) *"Consolidated bioprocessing of cellulosic biomass: an update"* **Current opinion in biotechnology *16***(5): 577–583. DOI: 10.1016/j.copbio.2005.08.009.

[40] Y. Liu, X. Xie, W. Liu, H. Xu, and Y. Cao, (2020) *"Consolidated bioprocess for bioethanol production from lignocellulosic biomass using Clostridium thermocellum DSM 1237"* **BioResources *15***(4): 8355. DOI: 10.15376/biores.15.4.8355-8368.

[41] A. Althuri, L. K. S. Gujjala, and R. Banerjee, (2017) *"Partially consolidated bioprocessing of mixed lignocellulosic feedstocks for ethanol production"* **Bioresource Technology *245***: 530–539. DOI: 10.1016/j.biortech.2017.08.140.

[42] S. Vaid, S. Sharma, H. C. Dutt, R. Mahajan, and B. K. Bajaj, (2021) *"One pot consolidated bioprocess for conversion of Saccharum spontaneum biomass to ethanol-biofuel"* **Energy Conversion and Management *250***: 114880. DOI: 10.1016/j.enconman.2021.114880.

[43] L. Chen, J.-L. Du, Y.-J. Zhan, J.-A. Li, R.-R. Zuo, and S. Tian, (2018) *"Consolidated bioprocessing for cellulosic ethanol conversion by cellulase–xylanase cell-surfaced yeast consortium"* **Preparative Biochemistry and Biotechnology *48***(7): 653–661. DOI: 10.1080/10826068.2018.1487846.

[44] L. R. Lynd, X. Liang, M. J. Biddy, A. Allee, H. Cai, T. Foust, M. E. Himmel, M. S. Laser, M. Wang, and C. E. Wyman, (2017) *"Cellulosic ethanol: status and innovation"* **Current opinion in biotechnology *45***: 202–211.

[45] A. Joshi, K. K. Verma, V. D Rajput, T. Minkina, and J. Arora, (2022) *"Recent advances in metabolic engineering of microorganisms for advancing lignocellulose-derived biofuels"* **Bioengineered *13***(4): 8135–8163.

[46] S. Kirdponpattara, S. Chuetor, M. Sriariyanun, and M. Phisalaphong, (2022) *"Bioethanol Production by Pichia stipitis Immobilized on Water Hyacinth and Thinshell Silk Cocoon"* **Applied Science and Engineering Progress *15***(3): 4662–4662. DOI: 10.14416/j.asep.2021.03.006.

[47] S. Lane, J. Dong, and Y.-S. Jin, (2018) *"Value-added biotransformation of cellulosic sugars by engineered Saccharomyces cerevisiae"* **Bioresource technology *260***: 380–394.

[48] M. Taha, M. Foda, E. Shahsavari, A. Aburto-Medina, E. Adetutu, and A. Ball, (2016) *"Commercial feasibility of lignocellulose biodegradation: possibilities and challenges"* **Current Opinion in Biotechnology *38***: 190–197.

[49] M. Broda, D. J. Yelle, and K. Serwańska, (2022) *"Bioethanol Production from Lignocellulosic Biomass—Challenges and Solutions"* **Molecules** ***27***(24): 8717.

[50] Z. Yao, Q. Wang, and Z. Dai, (2022) *"Recent advances in directed yeast genome evolution"* **Journal of Fungi** ***8***(6): 635. DOI: 10.3390/jof8060635.

[51] Y. A. Menegon, J. Gross, and A. P. Jacobus, (2022) *"How adaptive laboratory evolution can boost yeast tolerance to lignocellulosic hydrolyses"* **Current Genetics** ***68***(3-4): 319–342. DOI: 10.1007/s00294-022-01237-z.

[52] A. P. Jacobus, J. Gross, J. H. Evans, S. R. Ceccato-Antonini, and A. K. Gombert, (2021) *"Saccharomyces cerevisiae strains used industrially for bioethanol production"* **Essays in Biochemistry** ***65***(2): 147–161. DOI: 10.1002/biot.202000142.

[53] A. P. Jacobus, J. Gross, J. H. Evans, S. R. Ceccato-Antonini, and A. K. Gombert, (2021) *"Saccharomyces cerevisiae strains used industrially for bioethanol production"* **Essays in Biochemistry** ***65***(2): 147–161. DOI: 10.1042/EBC20200160.

[54] J. Sharma, V. Kumar, R. Prasad, and N. A. Gaur, (2022) *"Engineering of Saccharomyces cerevisiae as a consolidated bioprocessing host to produce cellulosic ethanol: Recent advancements and current challenges"* **Biotechnology Advances**: 107925.

[55] D. P. Procópio, E. Kendrick, R. Goldbeck, A. R. d. L. Damasio, T. T. Franco, D. J. Leak, Y.-S. Jin, and T. O. Basso, (2022) *"Xylo-Oligosaccharide Utilization by Engineered Saccharomyces cerevisiae to Produce Ethanol"* **Frontiers in Bioengineering and Biotechnology** ***10***: 105. DOI: 10.3389/fbioe.2022.825981.

[56] A. Madhavan, K. Arun, R. Sindhu, B. G. Nair, A. Pandey, M. K. Awasthi, G. Szakacs, and P. Binod, (2022) *"Design and genome engineering of microbial cell factories for efficient conversion of lignocellulose to fuel"* **Bioresource Technology**: 128555.

[57] F. Maleki, M. Changizian, N. Zolfaghari, S. Rajaei, K. A. Noghabi, and H. S. Zahiri, (2021) *"Consolidated bioprocessing for bioethanol production by metabolically engineered Bacillus subtilis strains"* **Scientific Reports** ***11***(1): 13731. DOI: 10.1038/s41598-021-92627-9.

[58] E. Guedon, M. Desvaux, and H. Petitdemange, (2002) *"Improvement of cellulolytic properties of Clostridium cellulolyticum by metabolic engineering"* **Applied and environmental microbiology** ***68***(1): 53–58. DOI: 10.1128/AEM.68.1.53-58.2002.

[59] S. Hon, D. G. Olson, E. K. Holwerda, A. A. Lanahan, S. J. Murphy, M. I. Maloney, T. Zheng, B. Papanek, A. M. Guss, and L. R. Lynd, (2017) *"The ethanol pathway from Thermoanaerobacterium saccharolyticum improves ethanol production in Clostridium thermocellum"* **Metabolic engineering** ***42***: 175–184. DOI: 10.1016/j.ymben.2017.06.011.

[60] R. Biswas, S. Prabhu, L. R. Lynd, and A. M. Guss, (2014) *"Increase in ethanol yield via elimination of lactate production in an ethanol-tolerant mutant of Clostridium thermocellum"* **PLoS One** ***9***(2): e86389. DOI: 10.1371/journal.pone.0086389.

[61] S. Kannuchamy, N. Mukund, and L. M. Saleena, (2016) *"Genetic engineering of Clostridium thermocellum DSM1313 for enhanced ethanol production"* **BMC biotechnology** ***16***(1): 1–6. DOI: 10.1186/s12896-016-0260-2.

[62] B. Dien, R. Hespell, L. Ingram, and R. Bothast, (1997) *"Conversion of corn milling fibrous co-products into ethanol by recombinant Escherichia coli strains K011 and SL40"* **World Journal of Microbiology and Biotechnology** ***13***: 619–625. DOI: 10.1023/A:1018554417934.

[63] C. Dong, J. Qiao, X. Wang, W. Sun, L. Chen, S. Li, K. Wu, L. Ma, and Y. Liu, (2020) *"Engineering Pichia pastoris with surface-display minicellulosomes for carboxymethyl cellulose hydrolysis and ethanol production"* **Biotechnology for biofuels** ***13***(1): 1–9. DOI: 10.1186/s13068-020-01749-1.

[64] S. Yanase, T. Hasunuma, R. Yamada, T. Tanaka, C. Ogino, H. Fukuda, and A. Kondo, (2010) *"Direct ethanol production from cellulosic materials at high temperature using the thermotolerant yeast Kluyveromyces marxianus displaying cellulolytic enzymes"* **Applied Microbiology and Biotechnology** ***88***: 381–388. DOI: 10.1007/s00253-010-2784-z.

[65] B. He, B. Hao, H. Yu, F. Tu, X. Wei, K. Xiong, Y. Zeng, H. Zeng, P. Liu, Y. Tu, et al., (2022) *"Double integrating XYL2 into engineered Saccharomyces cerevisiae strains for consistently enhanced bioethanol production by effective xylose and hexose co-consumption of steam-exploded lignocellulose in bioenergy crops"* **Renewable Energy** ***186***: 341–349. DOI: 10.1016/j.renene.2021.12.103.

[66] R. A. Cripwell, S. H. Rose, L. Favaro, and W. H. Van Zyl, (2019) *"Construction of industrial Saccharomyces cerevisiae strains for the efficient consolidated bioprocessing of raw starch"* **Biotechnology for biofuels** ***12***: 1–16. DOI: 10.1186/s13068-019-1541-5.

[67] J. K. Ko, Y. Um, H. M. Woo, K. H. Kim, and S.-M. Lee, (2016) *"Ethanol production from lignocellulosic hydrolysates using engineered Saccharomyces cerevisiae harboring xylose isomerase-based pathway"* **Bioresource Technology *209***: 290–296. DOI: 10.1016/j.biortech.2016.02.124.

[68] J. T. Cunha, T. Q. Aguiar, A. Romanı, C. Oliveira, and L. Domingues, (2015) *"Contribution of PRS3, RPB4 and ZWF1 to the resistance of industrial Saccharomyces cerevisiae CCUG53310 and PE-2 strains to lignocellulosic hydrolysate-derived inhibitors"* **Bioresource Technology *191***: 7–16. DOI: 10.1016/j.biortech.2015.05.006.

[69] E. J. Oh, N. Wei, S. Kwak, H. Kim, and Y.-S. Jin, (2019) *"Overexpression of RCK1 improves acetic acid tolerance in Saccharomyces cerevisiae"* **Journal of Biotechnology *292***: 1–4. DOI: 10.1016/j.jbiotec.2018.12.013.

[70] J. T. Cunha, C. E. Costa, L. Ferraz, A. Romanı, B. Johansson, I. Sá-Correia, and L. Domingues, (2018) *"HAA1 and PRS3 overexpression boosts yeast tolerance towards acetic acid improving xylose or glucose consumption: unravelling the underlying mechanisms"* **Applied microbiology and biotechnology *102***: 4589–4600. DOI: 10.1007/s00253-018-8955-z.

[71] H.-S. Kim, N.-R. Kim, J. Yang, and W. Choi, (2011) *"Identification of novel genes responsible for ethanol and/or thermotolerance by transposon mutagenesis in Saccharomyces cerevisiae"* **Applied microbiology and biotechnology *91***: 1159–1172. DOI: 10.1007/s00253-011-3298-z.

[72] K. Deanda, M. Zhang, C. Eddy, and S. Picataggio, (1996) *"Development of an arabinose-fermenting Zymomonas mobilis strain by metabolic pathway engineering"* **Applied and Environmental Microbiology *62***(12): 4465–4470. DOI: 10.1128/aem.62.12.4465-4470.1996.

[73] M. Zhang, C. Eddy, K. Deanda, M. Finkelstein, and S. Picataggio, (1995) *"Metabolic engineering of a pentose metabolism pathway in ethanologenic Zymomonas mobilis"* **Science *267***(5195): 240–243. DOI: 10.1126/science.267.5195.240.

[74] E. Joachimsthal, K. D. Haggett, and P. L. Rogers. "Evaluation of recombinant strains of Zymomonas mobilis for ethanol production from glucose/xylose media". In: *Twentieth Symposium on Biotechnology for Fuels and Chemicals: Presented as Volumes 77–79 of Applied Biochemistry and Biotechnology Proceedings of the Twentieth Symposium on Biotechnology for Fuels and Chemicals Held May 3–7, 1998, Gatlinburg, Tennessee*. Springer. 1999, 147–157.

[75] S. R. Kim, Y.-C. Park, Y.-S. Jin, and J.-H. Seo, (2013) *"Strain engineering of Saccharomyces cerevisiae for enhanced xylose metabolism"* **Biotechnology advances *31***(6): 851–861. DOI: 10.1016/j.biotechadv.2013.03.004.

[76] Y.-S. Jin, H. Ni, J. M. Laplaza, and T. W. Jeffries, (2003) *"Optimal growth and ethanol production from xylose by recombinant Saccharomyces cerevisiae require moderate D-xylulokinase activity"* **Applied and environmental Microbiology *69***(1): 495–503. DOI: 10.1128/AEM.69.1.495-503.2003.

[77] J. Lou, J. Wang, Y. Yang, Q. Yang, R. Li, M. Hu, Q. He, J. Du, X. Wang, M. Li, et al., (2021) *"Development and characterization of efficient xylose utilization strains of Zymomonas mobilis"* **Biotechnology for biofuels *14***(1): 1–18. DOI: 10.1186/s13068-021-02082-x.

[78] Y.-J. Liu, B. Li, Y. Feng, and Q. Cui, (2020) *"Consolidated bio-saccharification: Leading lignocellulose bioconversion into the real world"* **Biotechnology advances *40***: 107535. DOI: 10.1016/j.biotechadv.2020.107535.

[79] R. Yamada, T. Hasunuma, and A. Kondo, (2013) *"Endowing non-cellulolytic microorganisms with cellulolytic activity aiming for consolidated bioprocessing"* **Biotechnology advances *31***(6): 754–763. DOI: 10.1016/j.biotechadv.2013.02.007.

[80] D. G. Olson, J. E. McBride, A. J. Shaw, and L. R. Lynd, (2012) *"Recent progress in consolidated bioprocessing"* **Current opinion in biotechnology *23***(3): 396–405. DOI: 10.1016/j.copbio.2011.11.026.

[81] W.-H. Lee and Y.-S. Jin, (2017) *"Improved ethanol production by engineered Saccharomyces cerevisiae expressing a mutated cellobiose transporter during simultaneous saccharification and fermentation"* **Journal of biotechnology *245***: 1–8. DOI: 10.1016/j.jbiotec.2017.01.018.

[82] A. Madhavan, K. Arun, R. Sindhu, J. Krishnamoorthy, R. Reshmy, R. Sirohi, A. Pugazhendi, M. K. Awasthi, G. Szakacs, and P. Binod, (2021) *"Customized yeast cell factories for biopharmaceuticals: From cell engineering to process scale up"* **Microbial Cell Factories *20***(1): 124. DOI: 10.1186/s12934-021-01617-z.

[83] B. Chen, H. L. Lee, Y. C. Heng, N. Chua, W. S. Teo, W. J. Choi, S. S. J. Leong, J. L. Foo, and M. W. Chang, (2018) *"Synthetic biology toolkits and applications in Saccharomyces cerevisiae"* **Biotechnology advances *36***(7): 1870–1881. DOI: 10.1016/j.biotechadv.2018.07.005.

[84] J. T. Cunha, D. G. Gomes, A. Romani, K. Inokuma, T. Hasunuma, A. Kondo, and L. Domingues, (2021) *"Cell surface engineering of Saccharomyces cerevisiae for simultaneous valorization of corn cob and cheese whey via ethanol production"* **Energy Conversion and Management *243***: 114359. DOI: 10.1016/j.enconman.2021.114359.

[85] E. Gnansounou and A. Dauriat, (2010) *"Techno-economic analysis of lignocellulosic ethanol: a review"* **Bioresource technology *101***(13): 4980–4991. DOI: 10.1016/j.biortech.2010.02.009.

[86] M. Galbe, P. Sassner, A. Wingren, and G. Zacchi, (2007) *"Process engineering economics of bioethanol production"* **Biofuels**: 303–327. DOI: 10.1007/10_2007_063.

[87] F. C. Barba, R. M. Rodrıguez-Jasso, R. K. Sukumaran, H. A. Ruiz, et al., (2022) *"High-solids loading processing for an integrated lignocellulosic biorefinery: Effects of transport phenomena and rheology–A review"* **Bioresource Technology**: 127044. DOI: 10.1016/j.biortech.2022.127044.

[88] H. J. Kadhum and G. S. Murthy, (2022) *"Novel system design for high solid lignocellulosic biomass conversion"* **Bioresource Technology *350***: 126897.

[89] A. S. da Silva, R. P. Espinheira, R. S. S. Teixeira, M. F. de Souza, V. Ferreira-Leitao, and E. P. Bon, (2020) *"Constraints and advances in high-solids enzymatic hydrolysis of lignocellulosic biomass: a critical review"* **Biotechnology for Biofuels *13***(1): 1–28. DOI: 10.1186/s13068-020-01697-w.

[90] S. Chuetor, E. J. Panakkal, T. Ruensodsai, K. Cheenkachorn, S. Kirdponpattara, Y.-S. Cheng, and M. Sriariyanun, (2022) *"Improvement of enzymatic saccharification and ethanol production from rice straw using recycled ionic liquid: The effect of anti-solvent mixture"* **Bioengineering *9***(3): 115. DOI: 10.3390/bioengineering9030115.

[91] M. Sriariyanun, N. Kitiborwornkul, P. Tantayotai, K. Rattanaporn, and P.-L. Show, (2022) *"One-pot ionic liquid-mediated bioprocess for pretreatment and enzymatic hydrolysis of rice straw with ionic liquid-tolerance bacterial cellulase"* **Bioengineering *9***(1): 17. DOI: 10.3390/bioengineering9010017.

[92] C. Turnbull, M. Lillemo, and T. A. Hvoslef-Eide, (2021) *"Global regulation of genetically modified crops amid the gene edited crop boom–a review"* **Frontiers in Plant Science *12***: 630396. DOI: 10.3389/fpls.2021.630396.