







Review

Biochemical and Biorefinery Platform for Second-Generation Bioethanol: Fermentative Strategies and Microorganisms

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Abstract: Bioethanol is the most commonly used biofuel. It is an alternative to replace fossil fuels in renewable energy; it can be produced from lignocellulosic feedstock using a biotechnological process. Their participation of microorganisms is crucial in the bioconversion process of fermentation for ethanol production and can involve bacteria, fungi, and yeasts. However, when working within bioethanol processes from lignocellulose feedstock, microorganisms face some challenges, such as high temperature, high solids content, and the ability to ferment sugars for high ethanol concentration. Such challenges will depend on operative strategies, such as simultaneous saccharification and fermentation, separate hydrolysis and fermentation, semi-simultaneous saccharification and fermentation, and consolidated bioprocessing; these are the most common configurations. This review presents different trends of the microbial role, biochemical application, and fermentation operative strategies for bioethanol production of the second generation.

Keywords: lignocellulosic biomass; *Saccharomyces cerevisiae*; thermotolerance; operational strategies; ethanol; microorganisms

1. Introduction

The high consumption of energy and fossil fuels is affecting the Earth, causing environmental problems like global warming and pollution. In this context, bioethanol is an interesting alternative to replace gasoline as biofuel [1].

Like agro-industrial waste, bioethanol can be obtained from lignocellulosic materials (LCMs). These LCMs contain cellulose as a significant component, hemicellulose, and lignin. Together with fuels, LCMs have the potential to be converted to obtain high-added-value products within a biorefinery concept that includes bioprocesses in a circular bioeconomy [2].

To make the lignocellulosic cell wall more accessible and obtain different components, pretreatment is required for the rupture of these polysaccharides and other compounds,

next to the enzymatic hydrolysis steps, where the polysaccharides are converted to fermentable monosaccharides, and fermentation is used to convert these sugars to ethanol (Please see (Figure 1). These processes can be biological, physical, chemical, or a combination of these methods [3]. To produce sugars that must be fermented to ethanol, lignocellulose polysaccharides must be saccharified by acids or enzymes. In the enzymatic hydrolysis stage, a pretreatment has to be implemented to make cellulose accessible to cellulase enzymes to convert fermented sugars. For the production process, to establish a feasible strategy for all the steps to reduce cost, time, and environmental problems, the fermentation step is necessary and consequently requires the conditions of high temperature for the optimum process of enzymatic hydrolysis as well as fermentative microorganisms for biotechnological operative strategies [4]. The objective of this review is to show the new advances in the conversion of fermentable sugars to bioethanol through the biochemical platform of biorefineries based on the responsible microorganisms and operational fermentation strategies.

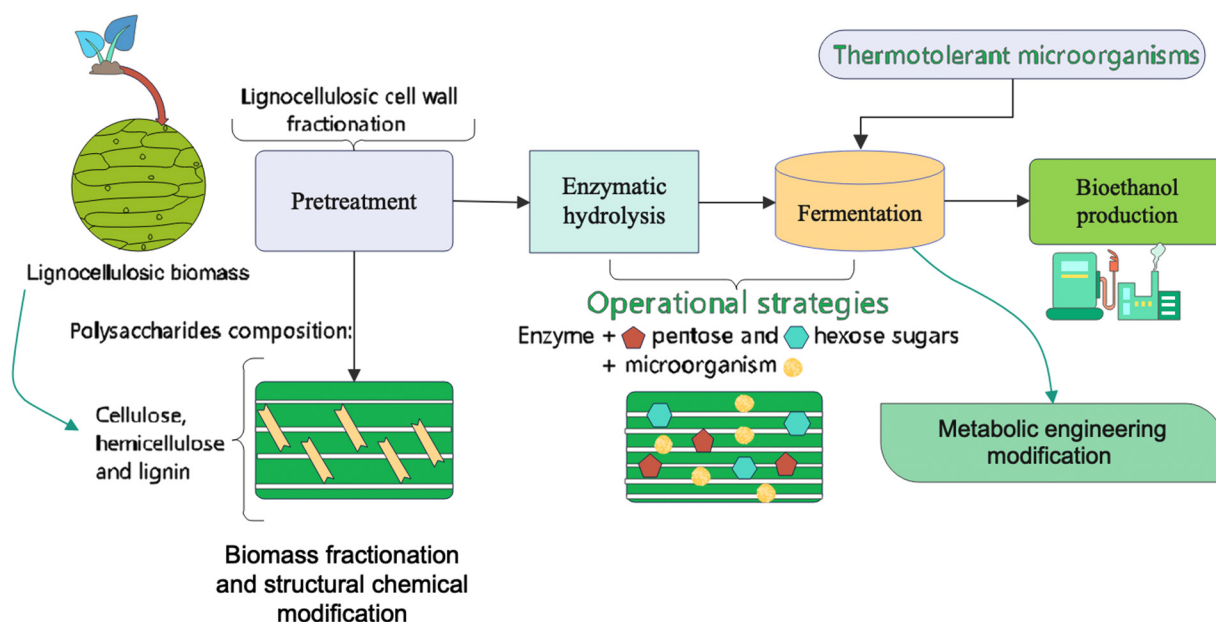


Figure 1. Bioethanol production from lignocellulosic biomass.

2. Bioethanol Production

Bioethanol is an alternative to be employed as a biodegradable liquid fuel. It contains 34.7% oxygen, in contrast to gasoline, where oxygen is absent; this reduces the amount of carbon monoxide emissions produced by different modes of transportation. This alternative biofuel is produced from fermentation [5].

Bioethanol of the second generation (2G) is used for alternative energy sources. It is made by the biological fermentation of sugars from lignocellulosic biomass feedstock to alcohol by microorganisms. The lignocellulosic bioethanol production process consists of the following steps: milling, pretreatment, enzymatic hydrolysis, fermentation, and distillation [6]. The principal bioethanol producing countries are Brazil and the U.S., which are linked to value compounds. Over the years, they have promoted programs to study and develop first-generation (1G) fuels from raw materials such as agro-industrial wastes like sugarcane, sorghum, etc. First-generation bioethanol has already been approved in the U.S., China, Canada, Brazil, and Sweden [5,7]. World production in 2021 was well over 15,015 million gallons; producing in the U.S. was 15,016 million gallons, and Brazil produced 7320 million gallons. Bioethanol production is expected to increase in the future [8–10].

Commercial 2G biorefinery plants are operating in different countries; for example, one plant run by Dupont Inc. is located in Nevada, U.S. This is a biorefinery cellulosic

ethanol plant using corn stover as feedstock. Another is operated by GranBio in Alagoas, Brazil; this biorefinery plant for 2G bioethanol uses bagasse and sugarcane straw; Enerkem Biofuels LP in Edmonton, Canada, operates another significant plant [8,11].

In 2021, the U.S. bioethanol production averaged 15 million gallons per year, with the U.S. ethanol production being 82% of the global total. The main feedstock of ethanol in the U.S. is corn; on average, corn production has increased in recent years, and this product has helped to increase ethanol production cost-effectively [8]. Biomass-derived fuel additives can be easily blended with gasoline to produce a fuel with fewer emissions [12].

Mexico can potentially produce bioenergy from different lignocellulosic materials; the most important crop residues are sugarcane bagasse, wheat straw, corncob, and corn stover [13]. The principal material is Agave, with a variety of around 200–300 species. *Agave tequilana* is a crop needing at least a 6-year life cycle before it is harvested. The principal production of the tequila beverage in Mexico is based on this crop, and it is estimated to use millions of tons of agave plants per year; that is why hundreds of tons of agave bagasse waste are generated annually [14]. A considerable amount of sugarcane bagasse, mainly produced in Latin America, is a profitable renewable agricultural resource for ethanol production.

To make a feasible process, biofuels' economic, social, and environmental aspects need to be designed and analyzed, and different techno-economic studies need to be evaluated [15]. The costs of converting LCMs to ethanol using biochemical technology are still high, but some innovative strategies to increase process yields and reduce production costs will be discussed hereafter [16,17].

For example, some of the techno-economic analyses of the production of 2G bioethanol have demonstrated that one of the raw materials that affect the final production cost of the bioethanol is the use of enzymes in the lignocellulosic hydrolysis process [18]. On the other hand, a life-cycle assessment of the production of lignocellulosic bioethanol suggests that integrated lignocellulosic enzyme production could be an option to reduce the cost of enzymes. Even integrated enzyme production can decrease greenhouse gas emissions during bioethanol production [19]. However, these analyses and improvements are necessary to reach higher enzyme productivity and yields to reduce the cost of production of lignocellulosic ethanol [20].

Different kinds of biomasses can be used for ethanol production in other companies and countries; these are divided into three generations [21]:

First generation: This generation is made from the use of grains and seeds, sugar feedstock such as cane juice, and raw materials such as corn and sugarcane; these materials compete with food, causing insufficient fuel demands [22].

Second generation: For 2G, the raw materials are agricultural residues (lignocellulosic biomass such as agave bagasse, corn residues, sugarcane bagasse, and wheat straw), wood, and cellulose paper (newspapers, recycled paper sludge). These produce biogas and alcohol [10].

Third generation: The third generation uses raw materials from aquatic biomass, like micro- and macroalgae. These raw materials are used for biofuel production and high-value-added compounds [4].

3. Ethanol-Producing Microorganisms

After the biomass enzymatic hydrolysis process, the next stage is ethanol production via the fermentation of sugars such as galactose, fructose, glucose, and mannose by microorganisms like yeasts and bacteria. In the case of cellulolytic enzymes, many microorganisms produce cellulases and xylanases, including the fungus and bacteria; for example, *Clostridium thermocellum*, *Trichoderma reesei*, *Aspergillus niger*, and *Aspergillus oryzae* are potential cellulase producers.

For bioethanol production, an ideal microorganism should support many factors that affect the production process, including pH variations, nutrient limitation, dissolved oxygen, and agitation speed. However, the most critical challenges are high tempera-

tures, high ethanol concentration, high pretreated solid loading, and inhibitors liberated in the pretreatment stage. In this sense, yeasts are more efficient for bioethanol production from LCMs than bacteria since they can tolerate high ethanol concentrations during fermentation. Industrial-scale fermentation for ethanol production would be more efficient if microorganisms (thermotolerant) could grow at high temperatures; publications on thermophilic microorganism have been increasing due to the different applications and industrial processes of these microorganisms [22,23].

3.1. Yeast

Yeasts are capable of fermenting different types of sugars. In particular, will-type *Saccharomyces cerevisiae* ferments glucose, and others such as *Scheffersomyces stipitis*, *Pachysolen tannophilus*, *Kluyveromyces marxianus*, and *Candida shehatae* can ferment xylose [5].

Yeast isolated from extreme environments can grow at high temperatures while producing ethanol. The optimal condition of growth temperature for yeasts ranges from 25 to 35 °C [24].

Characteristics of Yeasts

There are two types of yeast: flocculant and non-flocculant; the difference is that yeast cells form cell aggregates during fermentation, and non-flocculant yeast does not form aggregates. Centrifugation can suspend non-flocculent yeast cells lying in the medium [25,26].

Flocculant yeast cells are asexual and lead to reversible cell aggregation, where yeast cells adhere to each other to form thousands of cells called floc. This is a calcium-dependent process; the high cell density system is used for separating yeast cells from the brewing industry. The advantages are the reuse of the cell biomass for long periods, higher productivity, protection against ethanol stress, and resistance to contamination by other microorganisms [5,27].

3.2. Challenges to Overcome for the Fermenting Microorganism (High Temperature, Inhibitors, Mix of Sugars)

Even though *S. cerevisiae* has been domesticated to produce ethanol from different carbon sources for millennia, fermenting lignocellulosic hydrolysates poses some challenges. Hydrolysates can be prepared from other raw materials and using different technologies (i.e., pretreatments), and they contain toxic compounds that inhibit yeast growth and ethanol production. Furthermore, the range of sugars in the hydrolysates is so wide that it often goes beyond the carbon sources that any ethanologenic organism can metabolize [22,28].

3.2.1. Thermotolerance Yeast

Thermotolerant yeasts are very advantageous in the simultaneous saccharification and fermentation (SSF) strategy because they work with the saccharification at high temperatures simultaneously with enzymes. Thermotolerant strains are capable of growing at or above 40 °C. Several yeasts perform at higher temperatures, in a range of 40–45 °C [29].

3.2.2. Inhibitors

The severe conditions required for disrupting the lignocellulosic matrix lead to partial degradation of hemicelluloses and lignin, with the consequent formation of by-products inhibitory to downstream biochemical processes [30,31].

The amount and functionalities of the formed inhibitory by-products depend on the pretreatment method and conditions. The inhibition phenomena can become even worse when high solid loadings increase the sugar titers in the streams or when the process waters are recirculated for economic reasons.

Some of the best-known inhibitory compounds are the furan aldehydes furfural and 5-hydroxymethylfurfural (HMF) as well as the aliphatic acids formic acid, levulinic acid, and acetic acid, as different kinds of phenolic compounds. HMF and furfural result from the

degradation of sugars as pentoses and hexoses under high temperatures and acid medium, and their primary degradation leads the formation of formic and levulinic acid [30,32]. Acetic acid is formed from the hydrolysis of the acetyl groups of hemicelluloses.

Phenolic compounds, such as *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, vanillin, coniferyl aldehyde, syringaldehyde, and ferulic acid, among others, result mainly from the depolymerization of lignin during pretreatment, and their amount and nature depend on the biomass source and operational conditions [33].

The aliphatic acids and furan aldehydes are present in the hydrolysate phase; therefore, their inhibitory potential is high, even if their toxicity is relatively low. Acetic acid, formic acid, and levulinic acid inhibit the fermentation of *S. cerevisiae* when their expected concentration in hydrolysates is reached. Furfural and HMF, despite having inhibitory action lower than, for instance, that of aromatic aldehydes, pose a significant contribution to microbial inhibition due to their high concentrations [34,35]. On the other hand, phenolics are present in low amounts in hydrolysates, but their inhibitory action is commonly stronger than aliphatic acids and furan aldehydes. For example, ferulic acid is inhibitory to *S. cerevisiae* already at 1.0 mM, which is two magnitudes lower than the typical concentration range of aliphatic acids [34].

The importance of other inhibitors, such as quinones [36], and small aliphatic aldehydes, which have previously been underestimated, is now well documented. It was recently shown that *p*-benzoquinone, formaldehyde, and phenolic compounds, such as coniferyl aldehyde, vanillin, and *p*-hydroxybenzaldehyde, have higher molar toxicity than most of the previously investigated inhibitors [31].

To counteract the inhibition phenomena, different strategies can be followed. Increasing the inoculum volume can decrease the inhibition compounds. Other microorganism-related alternatives are the selection of microbial strains tolerant to specific inhibitors, evolutionary engineering, or metabolic engineering to improve the inhibitor resistance of yeast. However, despite the enhanced resistance of selected, evolutionary, or metabolically engineered strains, their performance often needs to be enhanced by established detoxification methods [37]. Although different detoxification approaches can effectively decrease the concentration of hydrolysate inhibitors, most require implementation in a separate process step, which poses economic restrictions. A promising method circumventing that problem is based on reducing agents, such as sodium sulfite and dithionite, which are compatible with yeast and cellulases.

3.2.3. Mix of Sugars

Different from the relatively homogeneous substrates used in the 1G ethanol processes, the lignocellulosic hydrolysates used to produce 2G ethanol are complex substrates containing hexoses, pentoses, methyl pentoses, and uronic acids. That different composition is challenging, since *S. cerevisiae* cannot utilize several of the sugars' hydrolysate phase of pretreatment. This problem is especially relevant for pentose sugar, such as xylose, the second most extensive sugar in the hydrolysates of hardwoods. Although there are microorganisms with the natural ability to ferment pentoses, they generally have low ethanol yield and are not inhibitor-tolerant. Therefore, in recent years, attention has been focused on developing recombinant xylose/fermenting strains of *S. cerevisiae* [38]. This is because the robustness, high ethanol yield, and productivity are more effective for ethanol production by microorganisms fermenting hexoses.

Different approaches have been developed to engineer yeast strains capable of converting xylose to ethanol, affecting the xylose metabolism and changing the intracellular redox balance [38]. While several of those approaches have led to results of academic interest, it is still necessary to develop studies for scale-up industrial bioprocesses, mainly the fermentation of xylose and glucose. Further investigation is required to create robust recombinant strains to efficiently ferment xylose to ethanol and all the lignocellulose-derived sugars at an industrial scale. Using non-*Saccharomyces* yeasts with industrially relevant properties is

necessary for research on cellulolytic and thermophilic tolerance, ethanol conversion, and cellulolytic bacteria to develop efficient ethanol fermentation strategies [39].

3.3. Genetically Modified Microorganisms

As previously mentioned, some challenges of bioethanol production can be addressed with genetically modified microorganisms.

Genetically engineered modification of strains of different microorganisms and bioprocess optimization have played essential roles in improved ethanol yields, increased pentose fermentation, and improved ethanol and inhibitor tolerance.

3.3.1. Yeast: *S. cerevisiae*

As highlighted previously, *S. cerevisiae* yeast is one of the selected microorganisms for ethanol production from lignocellulose biomass; this has been demonstrated to be highly tolerant towards derived inhibitors. However, several key issues must be considered to make *S. cerevisiae* completely effective for producing lignocellulosic ethanol at a commercial scale. First, the development of potent mixed sugar (mainly xylose) co-fermenting strains should be addressed. Secondly, robust strains tolerant to a wide range of degradation compounds should be obtained. Finally, developing *S. cerevisiae* strains capable of hydrolyzing the lignocellulosic substrate and directly fermenting the sugars through a consolidated bioprocessing (CBP) approach is of utmost importance.

Enhancing and improving xylose fermentation: Two different metabolic pathways for xylose assimilation have been introduced by *S. cerevisiae*: the oxidoreductive pathway, which involves xylose reductase (XR) and xylitol dehydrogenase (XDH) enzymes, and the xylose isomerase (XI). In both pathways, xylulose kinase (XK) phosphorylates xylulose, which is metabolized by the pentose phosphate pathway (PPP). XYL1 and XYL2 genes, involved in the XR/XDH pathway, can be cloned in *S. cerevisiae*. Nevertheless, the preference of these enzymes are translated into co-factors that cannot balance, causing xylitol accumulation. Altering the co-enzyme selection of XR and XDH is one of the most effective developments to decrease xylitol production and obtain high ethanol yields, as shown in Table 1.

The expression of XI from the mammal gut Bacteroidetes group (e.g., *Bacteroides valgutus* and *Alistipes* sp. HGB5) was obtained in *S. cerevisiae*, which results in this strain being capable of fermenting xylose as XI in bacteria and capable of converting xylose into 5-xylulose [40]. An evolutionary engineering strategy was recently performed to select top-performing XIs introduced in *S. cerevisiae* IR-2. This new strategy significantly improved xylose fermentation [41].

However, a recent study demonstrated that xylose fermentation was found in recombinant microorganisms simultaneously expressing both XI and XR/XDH pathways. In this case, the co-expression of both pathways significantly improved bioethanol production from non-detoxified hemicellulose hydrolysates [42].

Independently of the pathway present in *S. cerevisiae*, xylose fermentation to ethanol is more rapid when the endogenous XKS1 gene encoding for XK is overexpressed [43].

Low xylose has been attributed to other constraints on xylose fermentation by recombinant modified *S. cerevisiae* strains. Thus, recent efforts have been made to increase intracellular xylose transport.

Improving robustness toward lignocellulosic inhibitors: The use of robust fermentation microorganisms is important for enhancing a competitive bioethanol production process. Furthermore, inhibitor tolerance is more important when xylose-fermenting strains are available. Several studies have already demonstrated that the xylose fermentation capacity of genetically modified *S. cerevisiae* strains is more affected by stressful conditions than glucose fermentation [27].

The literature provides many examples of the use of evolutionary engineering, short-term adaptation, ethyl methanesulfonate (EMS) mutagenesis, and genome shuffling approaches to increase the tolerance of *S. cerevisiae* strains to different lignocellulosic degra-

dation compounds and make them robust [44]. Moreover, adaptive evolution, metabolic engineering strategies, and the expression of genes that encode enzymes that can resist specific inhibitors have also been reported to improve tolerance to lignocellulosic degradation compounds (Table 1).

Hydrolyzing *S. cerevisiae* strains for CBP: As stated in this chapter, CBP combines hydrolysis, enzyme production, and fermentation into a single stage. This strategy enhances the efficiency of processing by excluding exogenous hydrolytic enzymes and reducing the inhibition of cellulases because the fermenting microorganism rapidly consumes sugars. At this moment, however, the type of microorganism capable of CBP with high efficiency for industrial bioethanol production has not been identified [44,45].

Genetic engineering for enhancing hydrolase activities is required to use *S. cerevisiae* as a suitable CBP microorganism, as shown in Table 1. Recently, a single hydrolase gene or a combination of such genes was expressed in *S. cerevisiae*. New promoters have also been applied to increase the expression of cellulase genes. In addition, high copies of cellulase genes have been transferred, and cellulosome expression has also been studied. The proposed engineering a CBP yeast to express multiple components of cellulosomes from different microorganisms is the most complex strategy, and displaying hemicellulases and cellulases on the yeast cell wall can represent a significant development in research on CBP strategy [45].

Table 1. Current metabolic engineering strategies to obtain efficient *S. cerevisiae* strains for bioethanol production.

Metabolic Engineering Strategies of <i>Saccharomyces cerevisiae</i>			
Aim		Strategy	Ref.
Improving xylose fermentation	To decrease xylitol formation	Incorporation of the NADH oxidase from <i>Lactococcus lactis</i> in the industrial polyploidy strain <i>S. cerevisiae</i> JHS200	[46]
		Structure-guided mutagenesis and directed evolution to provide a compilation of variants of XR and XDH with altered co-factor preferences	[47]
	To increase xylose uptake/transport	Directed evolution of a glucose/xylose co-transporter from <i>Candida intermedia</i> significantly increases the xylose transport capacity in <i>S. cerevisiae</i>	[47,48]
		Mutation in a general co-repressor of CYC8 (Y353C) to modify hexose transporter expression and improve xylose metabolism in <i>S. cerevisiae</i>	[49]
		Discovery of new xylose transporters, such as the ones identified in <i>Aspergillus niger</i> and <i>T. reesei</i>	[50]
		Evolutionary engineering in engineered <i>S. cerevisiae</i> harboring genes for XR, XDH, and XK resulted in a mutation of chimeric transporter Hxt36p with an enhanced xylose uptake rate	[41]
		Evolutionary engineering to obtain mutant HXT7(F79S), with a few single nucleotide polymorphisms showing improved xylose uptake rates	[21,51]
		Incorporation of xylose transporter genes AT5G17010 and AT5G59250 from <i>Arabidopsis thaliana</i> to improve xylose transporter efficiency	[50,52]
Improving robustness toward lignocellulosic inhibitors	Modulation of spermidine (SPD) content by altered expression levels of the genes in the SPD biosynthetic pathway	[53,54]	
	Overexpression of WHI2 in engineered yeast significantly improved glucose and xylose fermentation under acetic acid stress	[55,56]	
	Disruption of the SIZ1 gene in <i>S. cerevisiae</i> increases furfural tolerance	[57]	
Obtaining hydrolyzing <i>S. cerevisiae</i> strains	Application of different yeast strains displaying a scaffoldin (mini CipA) and containing three cohesin domains, endoglucanase (CelA), exoglucanase (CBHII), or β -glucosidase (BGLI)	[58,59]	
	Expression of two endoglucanases from <i>T. reesei</i> (Cel7B and Cel5A) and cellobiohydrolases from <i>T. reesei</i> , <i>Aspergillus niger</i> , and <i>Phaenoraete chrysosporium</i> in <i>S. cerevisiae</i> Y294	[60,61]	
	Multiple copy integration of cellulase genes into the delta (δ) repeat sites of transposable elements (Tn) in the <i>S. cerevisiae</i> chromosome	[62]	
	Use of constitutive promoters, such as TEF1 and PGK1, to significantly increase cellulase expression in <i>S. cerevisiae</i>	[63]	
	Assembly of trifunctional mini cellulosomes containing a mini-scaffoldinin <i>S. cerevisiae</i>	[64]	

3.3.2. Non-Conventional Yeast Species

Besides *S. cerevisiae*, many non-conventional yeasts possess interesting data that could help bioethanol production, such as tolerance to inhibitors, high temperatures, osmosis, pentose sugar conversion, etc. However, more genetic tools are needed to make non-conventional yeast an appropriate platform to overcome all challenges related to lignocellulosic bioethanol production. The coming years will be crucial for developing rapid advances in synthetic/system biology techniques for industrial use of non-conventional yeasts. Recent efforts to produce non-conventional yeast strains to produce biofuels involve, in some cases, the adaptation of genetic tools previously applied to *S. cerevisiae*. This context has addressed many steps to modify the thermotolerant yeast *K. marxianus*. *K. marxianus* was engineered with simultaneous xylose and glucose fermentation [65]. In the same context, *K. marxianus* was genetically modified for pyruvate production with simultaneous xylose and glucose consumption [9]. The metabolic response of *K. marxianus* to xylose, including transcriptional profiles and evidence for organelle contributions, has also been modeled [66].

Besides *K. marxianus*, genetic modification of *Scheffersomyces stipitis* has received significant attention; the first genome-scale metabolic models of both *Komagataella pastoris* (iLC915) and *S. stipitis* (iSS884) were validated, which are very useful tools to study the potential of these yeasts [67,68].

3.3.3. Bacteria

Among the promising bacteria for bioethanol production are thermophilic organisms such as *Thermoanaerobacter ethanolicus*, *Thermoanaerobacterium saccharolyticum*, and *Clostridium thermocellum*, as well as *Escherichia coli* and *Zymomonas mobilis*. These have different advantages compared to *S. cerevisiae*, such as being thermotolerant (more than 70 °C), the ability to ferment several sugars, enzyme activity, and susceptibility to genetic modifications. However, there are also some disadvantages, as they all, except *Zymomonas*, have a low tolerance to ethanol, present catabolic repression, produce organic acids, and are sensitive to inhibitors [69].

C. thermocellum breaks cellulose to produce cellobiose and cellobiose. However, this microorganism does not use hemicellulose. Some systems, which will be explained later as CBP, use co-cultures with microorganisms that can ferment sugars of five carbons and are tolerant to alcohol.

In the production of cellulolytic enzymes, the routes of organic acid and the production of hydrogen (in the case of anaerobic bacteria) are sought to direct the flow of carbon to produce ethanol. It has been reported that blocking the lactate route increases ethanol production by 30% in *C. thermocellum* ATCC27405 [70].

Thermophilic bacteria, specifically *C. thermocellum*, secrete the enzymes necessary for the saccharification of cellulose in the form of a “cellulosome”, a complex of proteins that interact with each other to hydrolyze cellulose. It is organized on scaffolds with cohesin modules to manage the holocellulolytic hydrolases, an anchor module, and another carbohydrate-binding module (CBM). An advantage of using this type of microorganism is that it can grow at temperatures of 55 to 60 °C. A diagram of this cellulosome is shown in Figure 2.

There are thermophilic hemicellulolytic organisms, such as *Thermoanaerobacterium saccharolyticum*, which grows in hemicellulose when producing xylanases. A genetically modified strain generates up to 90% ethanol, based on the maximum theoretical value. Another species of this genus, *Thermoanaerobacterium calidifontis*, produces xylanase and ferments xylose and glucose into ethanol. It has been found that the *adhE* gene is necessary for ethanol production. It is proposed that the expression of the *CipA* gene of *C. thermocellum* could express cellulosomes in *T. saccharolyticum* for cellulose transformation [71].

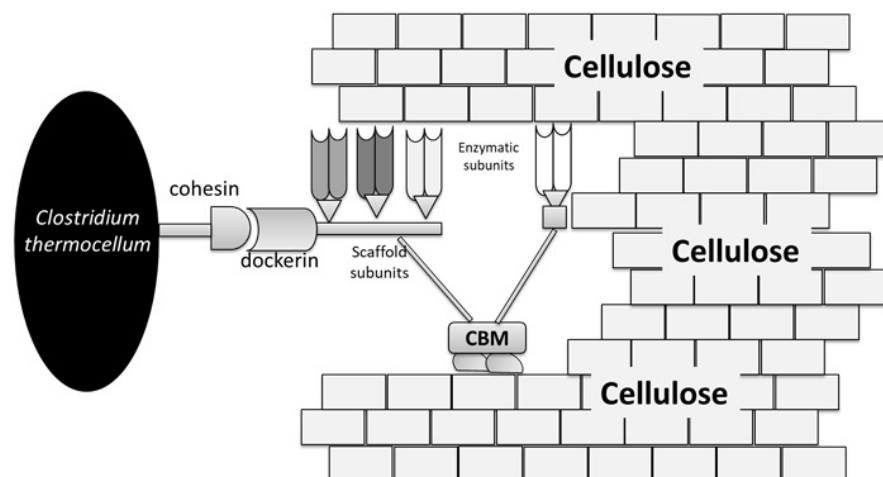


Figure 2. *Clostridium thermocellum* cellulosome.

Likewise, organisms capable of fermenting mono- and oligosaccharides, both pentose and hexoses, are sought. A strain of *Geobacillus thermoglucosidasius* (TM242) was modified to eliminate the expression of pyruvate dehydrogenase. There was a faster fermentation of glucose, where it was demonstrated that the metabolic flux for pyruvate and lactate were inhibited, and with the gene expression of the pyruvate decarboxylase gene *Gluconobacter oxydans*, *G. thermoglucosidasius* improved ethanol yield [72]. A brief table summarizing these microorganisms is provided below (Please see (Table 2)) [73].

Table 2. Ethanol-producing microorganisms and used for genetic engineering in bioethanol production.

Type	Microorganism	Characteristics	References
Yeasts	<i>Saccharomyces cerevisiae</i>	Facultative anaerobic yeast, high tolerance to ethanol	[1]
	<i>Kluyveromyces marxianus</i>	Thermophilic yeast is able to grow at high temperatures of 52 °C	[66]
	<i>Candida</i> sp.	Ethanologenic yeast, ferments xylose	[28]
Bacteria	<i>Komagataella pastoris</i>	Possesses cellulase enzymes favorable to the SSF process	[74]
	<i>Zymomonas mobilis</i>	Ethanologenic, high ethanol productivity	[75]
	<i>Clostridium thermocellum</i>	Suitable for CBP Processing	
	<i>Geobacillus thermoglucosidasius</i>	Ferments hexose, pentose, and oligomers	
	<i>Clostridium cellulovorans</i>	Amenability for genetic modification	[45,76]
Thermophilic bacteria	<i>Clostridium phytofermentans</i>	Secretes individual enzymes instead of cellulosomes.	
	<i>Thermoanaerobacterium calidifontis</i>	Hemicellulolytic, xylanases	
	<i>Thermoanaerobacterium saccharolyticum</i>	Resistance to extremely high-temperature, ferments xylan	[77]
	<i>Caldicellulosiruptor bescii</i>	Resistance to an extremely high-temperature of 70 °C	[77,78]
Fungi	<i>Thermoanaerobacter ethanolicus</i>	Have cellulolytic activity under high temperature	
	<i>Trichoderma reesei</i>	Produce high-level cellulase	[63]
	<i>Aspergillus niger</i>	Ability to produce plant biomass-degrading enzymes	[79]

3.3.4. Fungi

Filamentous fungi are naturally equipped with the enzymatic machinery to hydrolyze lignocellulosic biomass. Some species can even produce bioethanol [3], though their productivity is low compared to yeasts like *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* [28].

Two genetic modification strategies have been applied to improve the production of 2G bioethanol in the fungal strains: (1) engineering of wild-type cellulolytic fungi and (2) engineering of wild-type ethanologenic microorganisms (Please see Figure 3).

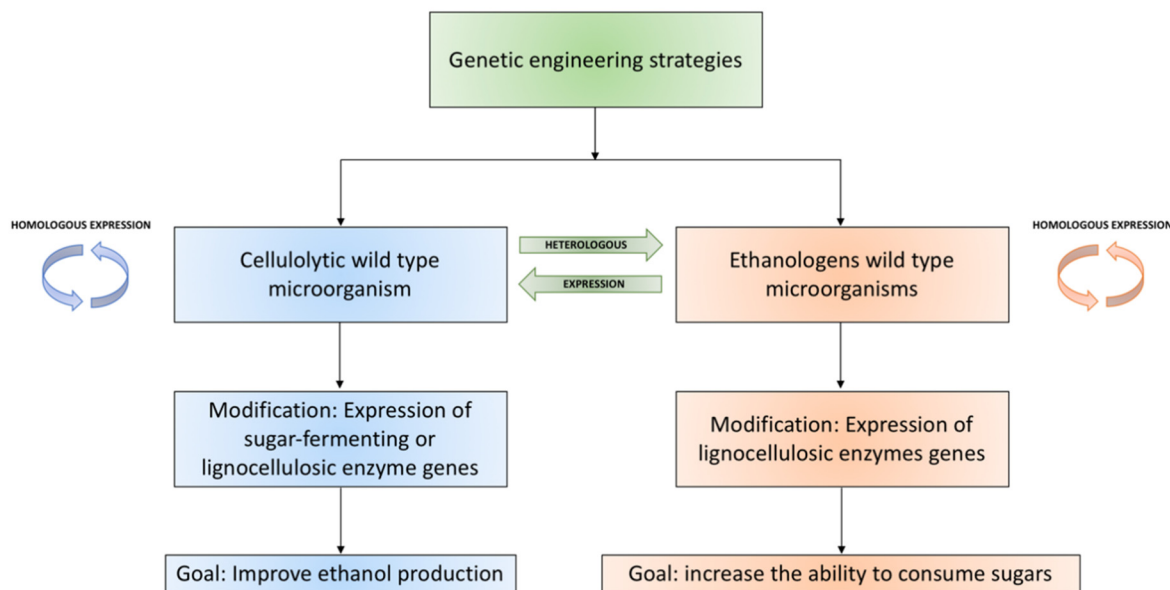


Figure 3. Genetic engineering strategies for enhanced 2G bioethanol production.

In the first case, lignocellulosic fungi with the capacity or not to produce bioethanol are genetically modified. This strategy introduces homologous or heterologous genes into the fungus to increase ethanol productivity (Table 3). On the other hand, the more common genetic modifications for ethanologenic fungi are the expression of lignocellulosic genes. These modifications increase the ability to consume different sugars as monosaccharides and polysaccharides (Table 3).

Table 3. Summary of some genetic engineering approaches for enhanced lignocellulosic enzymes and bioethanol production.

Fungal Strain (Receiver Organism)	Genetic Modification	Remarks	References
<i>Gloeophyllum trabeum</i> KU-41	Homologous overexpression of an endogenous gene encoding a putative laccase activity gene (Gtlcc3)	Clone <i>G. trabeum</i> L#61 showed higher laccase activity (2.7% of lignin degradation) than the <i>G. trabeum</i> KU-41 and 45% more ethanol production than the wild type	[80,81]
<i>Phanerochaete sordida</i> YK-624	Homologous overexpression of an extra pyruvate decarboxylase gene	Clone <i>P. sordida</i> GP7 produced 1.41 times more ethanol than the wild-type <i>P. sordida</i> YK-624	[82]
<i>Phanerochaete chrysosporium</i>	Constitutive co-expression of 4 oxidoreductases: manganese peroxidase (MnP), lignin peroxidase (LiP), versatile peroxidase (VP) from <i>Trametes versicolor</i> , and laccase (Lac) from <i>P. eryngii</i>	Constitutive co-expression of four oxidoreductases in a basidiomycete <i>P. chrysosporium</i> in functional form (culture in minimal medium)	[83,84]
<i>Phanerochaete chrysosporium</i>	Constitutive co-overexpression of 4 oxidoreductases: MnP, LiP, VP, and Lac from <i>Pleurotus eryngii</i>	Lignin depolymerization of sugarcane bagasse and wheat straw was enhanced by up to 25% in the presence of recombinant fungi in comparison with the wild-type strain. Sugar release on lignocellulose was 2- to 6-fold higher by recombinant fungi as compared with the control	[84]

Table 3. Cont.

Fungal Strain (Receiver Organism)	Genetic Modification	Remarks	References
<i>Trichoderma reesei</i>	Double overexpression of endogenous <i>egl2</i> and <i>bgl1</i> genes (endoglucanase and β -glucosidase enzymes) using <i>pyrG</i> marker (orotidine-5'-monophosphate decarboxylase gene)	The EG2–BGL1 double overexpression strain QEB4 displayed a remarkable enhancement of cellulolytic ability on pretreated corncob residues. Cellulose conversion (94.2%) was found for the delignified corncob residues after 48 h enzymatic saccharification	[85,86]
<i>Thermothelomyces thermophilus</i>	Heterologous expression of an alcohol dehydrogenase (ScAdh1) and cellodextrin transporters CDT-1/-2	The engineered strain JY518 increased ethanol production by 200% from cellobiose compared to the wild-type strain	[87]

In recent years, biorefineries designed to produce 2G bioethanol and high-value metabolites have taken advantage of the biotechnological versatility of fungi, as these microorganisms are indispensable in several industrial bioprocesses and they play a crucial role in biorefineries for the production of lignocellulosic enzymes. For example, some of the techno-economic analyses of the production of 2G bioethanol have demonstrated that one of the raw materials affecting bioethanol’s final production cost is the use of enzymes in the lignocellulosic hydrolysis process [88]. On the other hand, life-cycle assessment studies on bioethanol production suggest that integrated lignocellulosic enzyme production could be an option to reduce the cost of production; integrated enzyme production can even decrease greenhouse gas emissions during bioethanol production [89]. However, these analyses conclude that significant improvements are necessary to reach higher enzyme productivity and yields and reduce the production cost of lignocellulosic ethanol. Consequently, one of the main focuses in the fungal genetic transformation is related to the generation of over-producer lignocellulolytic fungi by using homologous and heterologous gene expression technology to produce mixed enzymatic extracts (native and recombinant enzymes) (Table 4).

Filamentous fungi are well known for being capable of producing lignocellulosic enzymes, not for being a producer of ethanol. However, this is common among certain species, such as *Lenzites betulinus*, *Fusarium oxysporum*, *Paecilomyces lilacinus*, and *Neurospora crassa*, among others. On the other hand, many studies using fungal strains for lignocellulosic bioethanol production have demonstrated the metabolic capabilities of these microorganisms; this knowledge has increasingly encouraged the use of modern molecular biology tools for the genetic transformation of fungi to increase or change the metabolic fluxes toward the production of bioethanol.

Table 4. Examples of the heterologous genetic modification of fungi.

Enzymes	Microorganism (Source of Gene)	Host Microorganism	References
β -glucosidase	<i>Penicillium decumbens</i>	<i>Trichoderma reesei</i>	[90]
Endoglucanases	<i>Trametes versicolor</i>	<i>Pichia pastoris</i>	[91]
Xylanase	<i>Orpinomyces</i> sp.	<i>Hypocrea jecorina</i>	[92]
Endo-1,4-xylanase	<i>Schizophyllum commune</i>	<i>Pichia pastoris</i>	[93]
Lacasse	<i>Pycnoporus cinnabarinus</i>	<i>Aspergillus niger</i>	[94]
Lacasse	<i>Trametes versicolor</i>	<i>Pichia methanolica</i>	[95]
Lacasse	<i>Trametes</i> sp.	<i>Trichoderma reesei</i>	[95,96]
Peroxidase	<i>Geotrichum candidum</i>	<i>Aspergillus oryzae</i>	[83]
Manganese peroxidase, Lignin peroxidase, Versatile peroxidase	<i>Trametes versicolor</i>	<i>Phanerochaete chrysosporium</i>	[97]
Lignin peroxidase	<i>Trametes versicolor</i>	<i>Phanerochaete chrysosporium</i>	[98,99]
Manganese peroxidase	<i>Pleurotus eryngii</i>	<i>Phanerochaete chrysosporium</i>	[82]
Cellulases	<i>Pyrococcus</i> sp.	<i>Talaromyces cellulolyticus</i>	[96,100]

4. Operative Strategies of Bioethanol Production

Generally, fermentation is performed by batch, fed-batch, and continuous modes according to the character of the microbial strain and expenditure of the process. Batch fermentation is a process used for ethanol production that involves only one-time initial feeding. It is associated with low cost, convenience, and flexibility. In contrast, continuous fermentation involves continuous feeding and removal of fermented broth containing a product, microbial cells, and undigested substrate at the same rate, leading to constant reaction volume in the fermenter. On the other hand, the fed-batch process uses the two modes of batch and continuous processes and the step-wise addition of the substrate into the fermenter with uniform feeding rates. Furthermore, different operating strategies are applied for ethanol production: simultaneous saccharification and fermentation (SSF); semi-simultaneous saccharification and fermentation (SSSF); separate hydrolysis and fermentation (SHF); simultaneous saccharification and co-fermentation (SSCF); simultaneous saccharification filtration and fermentation; and direct microbial conversion (DMC)/consolidated bioprocessing (CBP) [101,102].

Figure 4 represents the different operating strategies. Enzymes are important in strategies for bioethanol production [69]. Some reported studies using different operating strategies are summarized in Table 5. The effectiveness of these operational strategies can be determined based on two parameters: ethanol productivity, i.e., product generated per unit time (g/L/h), and ethanol yield, i.e., product developed per unit mass of solid substrate (g/g). Ethanol concentration is also important, as distillation costs are inversely proportional to the final ethanol concentration.

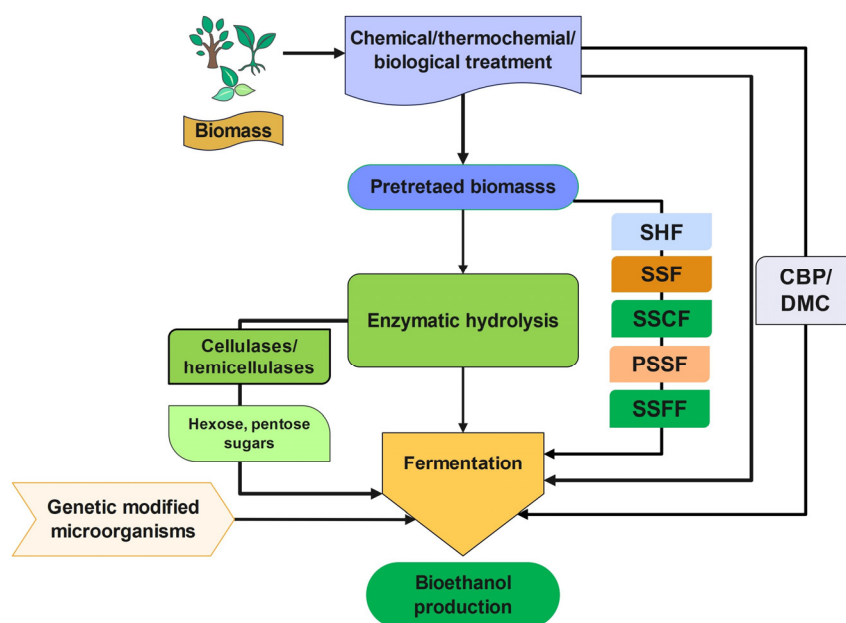


Figure 4. Operating strategies of ethanol production from lignocellulosic biomass (SHF: Separate Hydrolysis and Fermentation; SSF: Simultaneous Saccharification and Fermentation; SSCF: Simultaneous Saccharification and Co-Fermentation; PSSF: Pre-Simultaneous Saccharification and Fermentation; SSFF: Simultaneous Saccharification Filtration and Fermentation; DMC/CBP: Direct Microbial Conversion/Consolidated Bioprocessing).

Different operating modes show better productivity and yields than others in different reported studies. Therefore, no definite conclusion is available on which strategy is better; in fact, one can select any of these operating modes according to their needs and conditions, for instance, type of feedstock, type of fermenting strain, finance, etc. Moreover, ethanol productivity can be improved by the recirculation of cells and immobilization [103].

Table 5. Studies on lignocellulosic ethanol production using various operational strategies. * NR—not reported.

Process	Substrate	Hydrolytic Enzyme	Enzyme Loading	Fermenting Strain	Temperature (°C)	Reaction Time (h)	Ethanol Concentration (g/L)	Ethanol Yield (g/g-Sugar)	References
SHF	Steam-exploded SCB	Cellulase—Celluclast derived from <i>Trichoderma reesei</i> ; β -Glucosidase Novozyme 188	Cellulase—10 FPU/g cellulose, β -glucosidase 5% of cellulase	<i>Saccharomyces cerevisiae</i> UFPEDA 1238	Hydrolysis—50 Fermentation—34	120	23.38	0.39	[104–106]
	Steam-exploded palm kernel cake	Galactomannan, Driselase from <i>Basidiomyces</i> sp.; β mannanase from <i>T. reesei</i> ; Cellulase—Cellic C Tec2	Galactomannan—26.5; Driselase—53.1 U; Cellic CTec2—10.4 FPU/g	<i>Geobacillus thermoglucosidasius</i> TM242	Hydrolysis—50 Fermentation—60	48	9.9	0.47	[105]
	Cassava bagasse	Amylolitic Crude extract from <i>Rhizopusoligo sporus</i>	1.5 U/mL	<i>S. cerevisiae</i>	Hydrolysis—50 Fermentation—32	24	39.5	0.45	[107]
	Food waste	Cellulases derived from <i>Aspergillus oryzae</i>	Glucoamylas—13.5 U; Amylase—0.4 U; Cellulase—1 U	<i>Zymomonas mobilis</i>	Hydrolysis—50 Fermentation—30	54	71.8	0.50	[108]
	KOH-pretreated corn cob	Endoxylanase derived from <i>Streptomyces thermovulgaris</i> TISTR1948; Commercial cellulase iKnowZyMe AC	22.04 FPU/g corn cob	<i>Candida glabrata</i> KY618709	Hydrolysis—50 Fermentation—40	168	21.92	0.37	[109]
SSF	NaOH-pretreated SCB	Cellulase	20 FPU	<i>S. cerevisiae</i>	30	120	11.810	-	[110]
	H ₂ SO ₄ -pretreated <i>Arundodonax</i>	Cellulase—CellicCTec2	0.6% (v/v)	<i>Escherichia coli</i> MS04	40	96	25	-	[111]
	Steam-exploded triticale straw	Spezyme® CP	15 FPU/g cellulose	<i>S. cerevisiae</i>	37	144	29.6	0.41	[112]
	Hydrothermolysis SCB	Cellulase—Accellerase 1500	30 FPU g/g cellulose	<i>Kluyveromyces marxianus</i> IMB3	45	72	29.2	0.30	[113]
SSF	Microwave NaOH-H ₂ SO ₄ -pretreated rice straw	Cellulase derived from <i>Bacillus subtilis</i> NA15	CMCase—1.46 U/mL FPase—0.43 U/mL β -glucosidase—0.12 U/mL	<i>S. cerevisiae</i>	30	48	25.2	0.38	[114]
	KOH-pretreated corn cob	Xylanase derived from <i>S. thermovulgaris</i> TISTR1948; Cellulase—iKnowZyMe AC	22.04 FPU/g corn cob	<i>C. glabrata</i> KY618709	40	72	31.32	0.27	[109]

Table 5. Cont.

Process	Substrate	Hydrolytic Enzyme	Enzyme Loading	Fermenting Strain	Temperature (°C)	Reaction Time (h)	Ethanol Concentration (g/L)	Ethanol Yield (g/g-Sugar)	References
SSSF	Water-microwave-pretreated oil palm fronds	Cellulase derived from <i>A. niger</i>	70 FPU/g db	<i>S. cerevisiae</i>	Hydrolysis—50 Fermentation—37	120	4.313	0.32	[115,116]
	Soda lignin obtained from the spent liquor of the soda pulping of cedar wood chips	Cellulase—Genencor GC220	10 FPU/g pulp	<i>S. cerevisiae</i>	Hydrolysis—50 Fermentation—38	156	49.4	0.33	[117]
	Liquid hot water-pretreated reed	Cellulase derived from <i>T. longbrachiatum</i>	30–40 FPU/g db	<i>S. cerevisiae</i>	Hydrolysis—50 Fermentation—36	78	39.4	-	[118]
SSCF	Wood dust-pretreated	Cellulases/hemicellulases derived from <i>A. niger</i> and <i>T. reesei</i>	NR	<i>Z. mobilis</i> BCRC 10809	Hydrolysis—50 Fermentation—30	14	0.51	0.18	[119]
	Hydrothermally pretreated wheat straw	Crude extract derived from <i>Fusarium oxysporum</i> ; Cellulase—Cellic Ctec2	Crude extract—0.7 FPU/g dm; Cellic Ctec2—7 FPU/g dm	<i>S. cerevisiae</i>	30	72	62	0.44	[120]
	Hydrothermally pretreated corn flour and corn stover	Glucoamylase—Spirizyme® Fuel; Cellulolase—Accellerase 1500	Spirizyme—75 FPU/g cellulose Accellerase 1500—5FPU/g cellulose	<i>S. cerevisiae</i>	38	72	130.2	-	[103]
	Steam-exploded corn stover	Celluclast, β-glucosidase—Novozyme 188	Celluclast—15 FPU/g dm β-glucosidase—25 IU/g db	<i>S. cerevisiae</i> TMB3400	35	96	17.2	0.33	[121]
H ₂ O ₂ -pretreated corn stover	<i>Trichoderma reesei</i> extracted cellulase	1% (v/v)	<i>S. cerevisiae</i> ; <i>C. tropicalis</i>	32	144	109.24	0.48	[38]	
Ethylenedi-amine-pretreated corn stover	Cellic Ctec2; Cellic Htec2	10 mg/g glucan	<i>S. cerevisiae</i> SyBE005	Prehydrolysis—50 Fermentation—34	108	37.8	0.38	[122]	
CBP	NaOH-pretreated SCB	-	-	<i>Phlebia</i> sp. MG—60	28	240	4.5	0.33	[45]
	Waste newspaper	Crude extract of <i>Phlebia</i> sp. MG—60	NR	<i>Phlebia</i> sp. MG—60	30	216	4.2	0.20	[123]
	H ₂ O ₂ -pretreated rice bran	Cellic Ctec2	6% w/w	<i>S. cerevisiae</i> M2n [TLG1-SFA1]	30	60	42.06	0.47	[124]
	Hydrothermally pretreated wheat straw	Crude extract derived from <i>F. oxysporum</i> F3; Cellic Ctec2	1.23 FPU/g dm; Cellic Ctec 2—7 FPU/g dm	<i>S. cerevisiae</i>	30	72	58	0.41	[125]

4.1. Separate Hydrolysis and Fermentation (SHF)

Separate hydrolysis and fermentation (SHF) is a conventional fermentation method involving saccharification (hydrolysis) of pretreated biomass with the help of hydrolytic enzymes such as cellulases/xylanases, to hydrolyze the polysaccharides to monosaccharides, mainly glucose and xylose. Residual biomass is separated by filtration, and sugar-rich hydrolysate is subjected to fermentation by fermentative microorganisms. Iram et al. [124], reported SHF using alkali-pretreated sugarcane bagasse (SCB) (2.5%, *w/v* NaOH, 10%, *w/v* solid loading, 121 °C, 30 min) via hydrolysis using cellulase loading at 2% (*w/v*) solid loading at a temperature of 50 °C for a time of 8 h with a maximum concentration of reducing sugars of 140 g/L, which were fermented by *S. cerevisiae* and *P. stipitis* separately at 30 °C, resulting in maximum ethanol yields of 0.49 after 96 h [124]. In SHF, both processes are carried out separately; for instance, *S. cerevisiae* grows best at 32 °C, and hydrolytic enzymes at 50 °C. Thus, each process parameter can be optimized in terms of temperature, pH, solid loading, enzyme loading, and inoculum size. Despite the simplicity, SHF is associated with many limitations, such as higher capital cost, more extensive processing time, i.e., 72–96 h for hydrolysis and 24–72 h for fermentation, and a higher risk of contamination [4,102].

Moreover, there is a feedback inhibition of sugars to the hydrolytic enzymes, leading to lesser sugar and ethanol yields. SHF is affected by many factors, such as enzyme loading, temperature, pH, solid loading, feedback inhibition, and reaction time. Sugar release can be enhanced by increasing the solid loading in batches, but it causes mixing and heat-transfer difficulty. The fed-batch mode can be employed to maintain the viscosity at a low level.

4.2. Simultaneous Saccharification and Fermentation (SSF)

Simultaneous saccharification and fermentation (SSF) is an advanced process, as it deals with both steps simultaneously, enzymatic hydrolysis and fermentation, in a single system, i.e., enzymes and microbial strains are put together into the same vessel [126,127]. SSF is an efficient process for higher ethanol yield, elimination of feedback inhibition, higher productivity, short processing time, reduced operation costs, and reduced contamination risk [15,22,111]. The main reasons for the higher efficiency of SSF are the immediate consumption of glucose released during enzymatic hydrolysis and conversion to ethanol by the fermenting strain, thus preventing the feedback inhibition caused by sugar accumulation as in SHF [4,111]. Thermophiles/thermotolerant-like *Clostridium acetobutylicum*, *C. thermosulfurogenes*, *Kluyveromyces marxianus*, *C. thermosaccharolyticum*, *Bacillus polymyxa*, and *Thermoanaerobacter ethanolicus* have the almost optimum temperature, similar to enzymatic temperatures, and thus can be used for SSF [69,128]. Another major limitation of the process is the difficulty recycling enzymes and fermenting strains due to permanent incorporation with the biomass. Unfortunately, both these factors cause significant expense in the process. While enzymes could still be recycled by desorption with surfactant addition, it is not possible to recycle microbial cells; these can be reused after immobilization on some support, which enables cells to be separated from biomass [25,34].

Ethanol yield can be further enhanced by using high ethanol-tolerant strains or additives like safflower oil cake to provide higher ethanol tolerance to cells and by optimizing the process parameters, such as enzymes, temperature, yeast, nutrients, composition, and incubation time [1]. SSF usually shows greater ethanol yields with higher solids loadings than SHF. Generally, sugar release increases with the increase in solid loading up to a certain level. After that, it starts declining, a phenomenon known as the “solids effect”, due to improper binding of the enzyme with the substrate, thus limiting mass transfer and reducing the release of glucose [4,129]. Glucose inhibition starts above 15% and inhibits yeast growth at 40% (*w/v*) glucose concentration [130]. Fed-batch SSF involving the smaller addition of substrate after a regular interval can be used to eliminate the ‘solid effect’ through frequent biomass degradation, reducing the viscosity and increasing the mass transfer, thus enhancing sugar release during hydrolysis [4,22].

Moreover, the enzyme loading should be cost-effective enough to interact with the maximum available substrate. Low enzyme loading leads to lower ethanol yield and

longer reaction time. Some fed-batch studies also deal with enzyme feeding [131]. It is reported that a 50% reduction in enzyme loading is feasible if associated with a 6–7% reduced product yield and a maximum 30% increase in the reaction time [132]. Further, it has been observed that enzyme feeding at 30% solid loading is better for higher ethanol productivity [133].

Furthermore, any deviation from the optimum pH range of microbial strains results in reduced ethanol production. The optimum pH for ethanol production is 4.0–5.5 during the process, and the pH becomes lower than 4.0 due to acetic acid accumulation, which extends the duration. A pH above 5.0 leads to butyric acid formation, decreasing ethanol yield [134]. Moreover, higher inoculum size increases the ethanol yield by reducing the lag phase to a certain level, and no significant effect is observed above that level. Supplementation of cells with growth medium enhances the ethanol yield; however, small inoculum without medium components is favored from the industrial point of view to make the process cost-effective. Some low-price medium supplements like safflower oil seed cakes, sunflower, soy flour, and wheat mash can be used for higher product yield.

4.3. Simultaneous Saccharification and Co-Fermentation (SSCF)

SSF is generally associated with the non-utilization of pentose sugars (mainly xylose) when working with hexose-utilizing strains or acid-pretreated biomass, even if using pentose-utilizing strains, thus leaving pentoses unutilized in the biomass. However, xylose (major pentose) composes 25–35% of the lignocellulosic structure. Therefore, while working with SSF, only cellulosic ethanol yield is achieved. Still, to make SSF more economical and favorable, pentose sugar, i.e., xylose, must also be utilized. Hence, maximum utilization of the LCB will further increase the ethanol yield up to 30–35% [111]. Thus, xylose is fermented along with glucose in a process called simultaneous saccharification and co-fermentation (SSCF) using naturally or genetically engineered pentose sugar-utilizing strains [135]. However, microorganisms utilize glucose before xylose, and a high glucose concentration inhibits xylose metabolism, as it competitively enters into the same transporter faster than xylose. Adaptation of continuous SSCF by keeping glucose concentration below 2.3 g/L leads to the complete utilization of xylose and glucose through the same transporters [136].

Further, pre-fermentation involving fermentation of free hexoses before enzyme loading also keeps glucose concentration at lower levels for enhanced xylose uptake. Furthermore, two-step SSCF, which involves hydrolysis and fermentation of xylan and xylose before glucose, improves the xylose utilization significantly, as the glucose release rate is lower in the first step in the process, which does not have any effect on the microbial consumption of xylose. Two-step SSCF also reduces the redox imbalance due to the presence of many nutrients and oxygen at the beginning, promotes xylose utilization, and avoids ethanol inhibition. Moreover, hexose- and pentose-utilizing strains must be compatible with ethanol tolerance. Instead, a single strain capable of operating glucose and xylose can also be used [137]. Unhydrolyzed solids of the substrate were also found to have an inhibitory effect on xylose consumption, but this problem could be avoided by using fed-batch SSCF by maintaining the low substrate levels [40].

Moreover, unhydrolyzed solids can be solubilized either by increasing the xylanase and pectinase loading in pre-hydrolysis or using pure endo-glucanase. Still, it will compromise the cost-effectiveness of the process [88]. SSCF is an outstanding approach for enhanced production of 2G ethanol over SHF and SSF, as it leads to almost total xylose consumption, resolving feedback inhibition and shortening process duration [138].

4.4. Semi or Pre-Simultaneous Saccharification and Fermentation (SSSF or PSSF)

Significant limitations of SHF and SSF are feedback inhibition in enzymatic hydrolysis and divergence of optimal temperature, respectively, which can be resolved by adopting another more advanced process: semi-simultaneous saccharification and fermentation (SSSF) or pre-simultaneous saccharification and fermentation (PSSF), which combine the advantages of both processes; the pre-hydrolysis step of SHF and carrying forward the unfiltered

substrate for fermentation, similar to SSF [139]. SSSF involves enzymatic pre-hydrolysis under optimal conditions with a faster hydrolytic rate for an appropriate time duration. Feedback inhibition is avoided by frequent fermentation of sugars to ethanol, thus resulting in higher productivity and yield than SSF and SHF. After the pre-hydrolysis step, SSF starts after an optimal period during equilibrium between inhibitory and rate-controlling factors [140,141]. Generally, in SSSF, pre-hydrolysis and fermentation temperatures are 50 °C and 30–37 °C, respectively [4,22]. However, SSSF needs an optimal time to initiate the fermentation to balance the inhibitory and rate-controlling factors. The optimum time has been recorded as 18–24 h for pre-hydrolysis and 48–72 h for fermentation [142]. Further, higher substrate concentration increases the inhibitory by-products, which leads to reduced ethanol yield. Thus, fed-batch SSSF improves ethanol production by maintaining the substrate loading at a constant level.

4.5. Simultaneous Saccharification, Filtration, and Fermentation (SSFF)

Despite eliminating the problem of feedback inhibition during enzymatic hydrolysis and using different optimal conditions for both the steps in SSSF, the issue of reusing of fermenting strain could be resolved by adopting a more advanced process: simultaneous saccharification, filtration, and fermentation (SSFF). In this process, pretreated biomass is enzymatically hydrolyzed, and a sugar-rich suspension is filtered through a cross-flow membrane and fermented to ethanol [143]. The fermentation medium is homogenized between the hydrolysis and fermentation system, i.e., residual biomass is kept in the hydrolysis vessel, and sugar-rich filtrate continuously feeds into the fermentation vessel. A flocculating strain of *S. cerevisiae* was successfully reused for five cultivations of SSFF and obtained an ethanol yield of 85% in SSFF. For fermenting, culture facilitates using a flocculating yeast strain. The capacity and life span of the filter membrane must be examined for a long time.

4.6. Direct Microbial Conversion/Consolidated Bioprocessing (DMC/CBP)

The hydrolysis step in fermentation is a major limitation of ethanol production, as hydrolytic enzymes are costly. This step cannot be avoided, and an alternative has been made available recently. However, combining the production of hydrolytic enzymes, the hydrolysis of polysaccharides in the cell wall of biomass, and ethanol production (sometimes biomass pretreatment also) in a single vessel or, more specifically, within a single microbial strain would be a better choice, named as direct microbial conversion (DMC) or consolidated bioprocessing (CBP) [144]. However, no naturally occurring microorganism is so perfect that it can fulfill all the requirements included in DMC/CBP, i.e., it can degrade LCB completely and utilize all the sugars released to produce ethanol. Therefore, they need to be modified through metabolic engineering. Thus, genetically engineered microorganisms (GMOs) with excellent hydrolytic and ethanologenic activities can be applied to convert biomass directly. This approach results in high ethanol yield, with less time leading to higher productivity, and reduces the cost of biomass processing and the number of fermenters needed due to the single system [144]. Sharma et al. [144] reported that GMO *C. phytofermentans* ATCC 700394 hydrolyzed 76% and 88.6% of glucan and xylan, respectively, of ammonia fiber explosion (AFEX)-pretreated corn stover in 10 days with an ethanol titer of 2.8 g/L, which was 71.8% of that observed by SSSF (3.9 g/L) separately. Moreover, the risk of contamination was also eliminated in this approach due to cellulose non-adherent contaminants that are competitively eliminated by desirable cellulose-adherent microbes using sugars released from biomass [144,145].

The strategy generally involves three steps: the production of cellulases/xylanases, hydrolysis of polysaccharides to sugars, and fermentation of glucose/xylose to ethanol. There are two approaches to modifying microorganisms: native cellulolytic and recombinant cellulolytic. The naturally occurring cellulolytic microorganism is engineered for elevated ethanol production by incorporating cellulose adherence and high specific and fermenting activity. In contrast, the recombinant cellulolytic approach involves engineering

non-cellulolytic but efficient ethanologen with cellulase/hemicellulase systems to perform the whole SSF process. Several anaerobic, cellulolytic, and thermophilic microbial strains, such as *Neurospora crassa*, *Monilia* sp., *C. thermocellum*, and *Paecilomyces* sp., are suitable for DMC/CBP. Thermophilic strain *Caldicellulosiruptor* sp. can directly hydrolyze the raw LCB without any pretreatment. In fact, *C. phytofermentans* ATCC 700394 is a promising native CBP strain, as it secretes the highest amount of cellulases/hemicellulases and can produce ethanol and acetate [146,147]. However, more work for this approach is necessary due to the need for suitable gene-transfer techniques.

One strategy known as electro transformation (ET) has been reported for *Candida cellulolyticum*, *C. thermocellum*, *Thermoanaerobacterium thermosaccharolyticum*, and *T. saccharolyticum* [1,147]. In a reported study, *T. saccharolyticum* was engineered by knocking out the genes for lactic acid production and expressing genes for pyruvate decarboxylase and alcohol dehydrogenase, which decreased lactate production, and increased growth, acetate, and ethanol production [146]. However, GMOs cannot tolerate toxic metabolites such as phenols, furfural, acetate, lactate, and formate. Thus, inhibitors and ethanol tolerance are known to be expressed in GMOs such as *S. cerevisiae*, *C. thermocellum*, *T. thermosaccharolyticum*, and *E. coli* [30]. Recently, a single hydrolase gene or combination of such genes has been expressed in *S. cerevisiae*. New promoters have also been applied to increase heterologous expression of cellulase genes. In addition, a high copy number of cellulase genes has been transferred, and cellulosome expression has also been studied. Enzymatic and microbial synergy also influences the efficiency of the process. Generally, on an industrial scale, cellulose hydrolysis is carried out by a cellulose–enzyme (CE) complex without the involvement of the cellulolytic strain. However, cellulose–enzyme microbe (CEM) complexes were found to hydrolyze cellulose more efficiently than the CE complex. Co-culturing strains like *S. cerevisiae* and *C. thermocellum*, which do not utilize xylose efficiently, with other bacteria such as *C. thermosaccharolyticum* is another research focus in CBP. During CBP, cell growth and enzyme production need nitrogen sources within the medium; for instance, *C. phytofermentans* uses amino acids/peptides provided in the medium.

Further, the process's lower initial pH (<6.0) leads to higher ethanol yield. Reduced CBP performance at pH 6.0 was associated with the poor growth of *C. phytofermentans*. However, it was found that pH 7.0 is optimum for DCM/CBP, with efficient glucan/xylan conversions [147]. Moreover, biomass particle size is inversely proportional to its surface area and rate of enzymatic hydrolysis. Although it is a promising approach for advancements over existing operating strategies, CBP needs more efficient techniques with optimum conditions for proficient, eco-friendly, and cost-effective 2G ethanol production.

5. Conclusions

The yeast *S. cerevisiae* is a robust microorganism that allows the fermentation of different sugars and shows adaptation to varying operating conditions such as temperature, substrate (sugar) concentration, inhibitor compounds, osmotic stress, and ethanol exposure. Also, the *S. cerevisiae* strain can have specific characteristics, such as flocculation; this property can mean a reduction in the costs of bioethanol production, avoiding or eliminating the centrifugation and recovery of the yeast. The mutant and genetically modified strains of microorganisms have played an important role in improving ethanol yields, a critical strategy today. On the other hand, the future perspectives for the production of the second generation of bioethanol, the capital cost, and the energy required for distillation are important elements to consider; therefore, increasing the final concentration of ethanol is an important goal to reduce the costs of down-stream processing (recovery and purification of ethanol) using different operational strategies for bioethanol production, such as simultaneous saccharification and fermentation, semi-simultaneous saccharification and fermentation, separate hydrolysis and fermentation, and consolidated bioprocessing. Moreover, studying these strategies at pilot and industrial scales is necessary.

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