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## Review

# Thermotolerant fermenting yeasts for simultaneous saccharification fermentation of lignocellulosic biomass



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## ABSTRACT

Lignocellulosic biomass is the most abundant renewable source of energy that has been widely explored as second-generation biofuel feedstock. Despite more than four decades of research, the process of ethanol production from lignocellulosic (LC) biomass remains economically unfeasible. This is due to the high cost of enzymes, end-product inhibition of enzymes, and the need for cost-intensive inputs associated with a separate hydrolysis and fermentation (SHF) process. Thermotolerant yeast strains that can undergo fermentation at temperatures above 40°C are suitable alternatives for developing the simultaneous saccharification and fermentation (SSF) process to overcome the limitations of SHF. This review describes the various approaches to screen and develop thermotolerant yeasts via genetic and metabolic engineering. The advantages and limitations of SSF at high temperatures are also discussed. A critical insight into the effect of high temperatures on yeast morphology and physiology is also included. This can improve our understanding of the development of thermotolerant yeast amenable to the SSF process to make LC ethanol production commercially viable.

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## Contents

1. Introduction . . . . .	83
2. LC biomass as substrate for ethanol production . . . . .	83
3. Processes of second-generation bioethanol production . . . . .	83
3.1. SHF process . . . . .	83
3.2. SSF process . . . . .	84
3.3. SSCF process . . . . .	84
3.4. CBP process . . . . .	85
3.5. SSFF process . . . . .	85
4. Screening of yeast strains suitable for fermentation at high temperatures . . . . .	85
5. Effect of high temperature on yeast . . . . .	86
6. Role of thermotolerant yeast in SSF . . . . .	87
7. Methods for developing yeast strains suitable for SSF . . . . .	88
7.1. Site-directed mutagenesis . . . . .	88
7.2. Genome shuffling approach . . . . .	88
7.3. Mutagenesis . . . . .	89
7.4. Metabolic engineering . . . . .	89
7.5. Cell encapsulation . . . . .	89
7.6. Physiological adaptation or evolutionary engineering . . . . .	89
8. Benefits of high-temperature fermentation using thermotolerant yeasts . . . . .	90
8.1. Cooling costs . . . . .	90
8.2. Cost reduction at the SSF stage . . . . .	90
9. Limitations associated with high-temperature fermentation . . . . .	90

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10. Conclusions and future perspectives . . . . .	90
Conflict of interest . . . . .	90
Financial support . . . . .	90
Acknowledgments . . . . .	90
References . . . . .	90

## 1. Introduction

Driven primarily by the global increase in energy consumption, depletion of fossil fuel reserves and concerns about climate change, new renewable and environment-friendly sources of energy are being explored. Plant biomass is the most abundant renewable source of energy. It can be used to produce second-generation biofuels; however, it is largely wasted either by burning or by disposal in landfill sites. This leads to the release of greenhouse gases, which has a harmful effect on the environment. The process of ethanol production from lignocellulosic (LC) biomass requires four main steps: i) pretreatment of LC biomass; ii) enzymatic saccharification of pretreated biomass to yield sugar monomers; iii) fermentation of hydrolyzed sugars to ethanol, butanol, etc. by fermenting organisms; and iv) distillation. The process is termed as separate hydrolysis and fermentation (SHF) when saccharification and fermentation are performed separately. Due to the high cost of the biomass-hydrolyzing enzymes (cellulases and hemicellulases) and pretreatment methods, the production of LC ethanol by SHF is not economically viable.

During enzymatic saccharification, the hydrolytic enzymes are subject to feedback inhibition due to the accumulation of sugar monomers and cellobiose in the medium. This in turn reduces the efficiency of these enzymes. This limitation can be overcome by a process known as simultaneous saccharification and fermentation (SSF). Here, saccharification and fermentation are performed simultaneously; thus, the hydrolyzed sugars are continuously converted into ethanol, thereby enhancing the efficiency of enzymatic saccharification in the absence of feedback inhibition. However, the major limitations of SSF are the different temperature optima of biomass-hydrolyzing enzymes (45–50°C) and the fermenting organisms (30°C). Therefore, there is a need to discover cold-adaptive hydrolytic enzymes and thermotolerant fermenting yeasts to develop economically viable SSF technology. In practice, it is very difficult to reduce the optimum temperature of cellulases via protein engineering. Therefore, identifying thermotolerant yeast with higher ethanol production efficiency can be a key breakthrough for the SSF process. The SSF by thermotolerant yeasts offer the following advantages in bioethanol production:

- Reduction in total number of steps, thereby lowering the utility requirement and reducing capital investment including equipment costs
- Reduction in contamination possibility by decreasing glucose concentration and ethanol production
- Improvement in efficiency of saccharification by alleviating feedback inhibition of cellulase
- Reduction in cooling cost, as chiller unit is not required
- Continuous ethanol evaporation from broth under reduced pressure
- Suitability for use in tropical regions with high temperatures

## 2. LC biomass as substrate for ethanol production

LC biomass refers to plant dry matter, which is mainly composed of carbohydrate polymer, cellulose (38–50%), hemicellulose (23–32%), and the aromatic polymer lignin (15–25%) [1]. It is the most abundant raw material for the production of ethanol. Every year,  $2 \times 10^{11}$  mt of LC biomass is produced globally,  $8\text{--}20 \times 10^9$  mt of which is potentially accessible for processing. Structurally, cellulose and

hemicellulose are closely linked to lignin, making the polysaccharides inaccessible for hydrolysis by cellulases and hemicellulases. Cellulose is a linear polymer of D-glucose joined by  $\beta(1,4)$  glycosidic linkages with reducing and nonreducing ends. Cellulose fibrils are arranged in parallel stacks with hydrogen bonding and weak van der Waals forces, forming cellulose microfibrils. These cellulose microfibrils have both crystalline and amorphous regions that are bound together by hemicellulose and lignin to form macrofibrils. The second most important fraction of LC biomass is hemicellulose, a heteropolymer of pentoses (xylose and arabinose) and hexoses (glucose, galactose, and mannose). Xylan, a  $\beta(1,4)$  linked xylose homopolymer, is a major hemicellulosic component of hardwood trees, whereas softwood primarily contains mannans and glucomannans [2]. Lignin, which provides rigidity to plants, is a heteropolymer of *p*-hydroxyphenyl, syringyl, guaiacyl, and syringyl monolignol units, which form a complex network around cellulosic microfibrils. LC biomass can be grouped under three different categories: virgin biomass, energy crops, and waste biomass. All terrestrial plants such as trees, bushes, grasses, and crop plants are collectively termed as virgin biomass. Waste biomass is the low-value by-product of virgin biomass such as corn stover, sugarcane bagasse, and saw mill and paper mill wastes. Energy crops such as switchgrass, elephant grass, cassava, and sweet sorghum, which produce more biomass, are cultivated for use as raw materials in ethanol production.

Cellulose and hemicellulose can be hydrolyzed by holocellulases to sugars. These in turn can be fermented to produce biofuel. However, lignin is a polyphenolic compound that cannot be fermented. Agricultural crop residues, industrial and urban waste, forestry residues, and dedicated energy crops such as switchgrass, giant reed, *Miscanthus*, poplar, and willow are the most widely used and abundant LC feedstocks. The proportion of constituents of LC feedstocks varies with the type of feedstock used. The residue from cultivable land can include straw from agricultural crops such as paddy and wheat; groundnut shells; corn stover; sunflower stalks; cotton stalks; grass fibers; and agricultural by-products such as corn cobs, sugarcane bagasse, palm mesocarp fibers, sunflower, and barley hulls. Rice husks and wheat bran arising from the processing of agricultural commodities can also be used as a substrate for LC ethanol production [3,4,5,6]. Forestry waste includes wood chips, slashes, branches of dead trees, hardwood, softwood, and tree prunings [7]. Processing papers, household wastes, cotton linters, pulps, food processing waste, and wastes from fruit and vegetable processing are categorized as industrial and urban waste [8,9].

## 3. Processes of second-generation bioethanol production

Scientists across the globe have developed different processes for ethanol production from LC biomass (Table 1). These processes include SHF; SSF: simultaneous saccharification and co-fermentation (SSCF); consolidated bioprocessing (CBP); and simultaneous saccharification, filtration, and fermentation (SSFF). Every process has its own advantages and limitations, which are listed in Table 2.

### 3.1. SHF process

The SHF process is the oldest method used to produce LC ethanol. In this process, externally produced enzyme cocktails are used to hydrolyze pretreated LC biomass to yield sugar monomers. The

**Table 1**

A brief overview of conditions used by researchers for bioethanol production.

Process	Yeast	Enzyme loading	Substrate loading	Temp. (°C)	% Theoretical yield	Reference
SHF	<i>S. cerevisiae</i>	50 FPU	11.25% w/v (0.651 g sugars/g dry substrate)	30	41.69	Sindhu et al. [10]
	<i>S. cerevisiae</i>	3 U/mL FPase and 9 U/mL $\beta$ -glucosidase	37.47 g/L sugars	30	96	Gupta et al. [11]
SSF	<i>K. marxianus</i> TISTR5925	-	96.2 g/L sugars	40	92.2	Murata et al. [12]
	<i>Blastobotrys adenivorans</i> RCKP-2012	22.5 FPU	8% w/v	50	46.87	Antil et al. [13]
	<i>K. marxianus</i>	-	10% w/v (380 mg sugars/g carrot pomace)	42	92	Yu et al. [14]
SSCF	<i>S. cerevisiae</i>	-	26% dry matter of pretreated wheat straw	30	67	Paschos et al. [15]
	<i>S. cerevisiae</i> KE6-12	9 FPU g <sup>-1</sup> WIS (water-insoluble solids)	7.9% WIS	30	77	Koppram et al. [16]
	<i>S. cerevisiae</i> TMB3400	Cellulase 30 FPU g <sup>-1</sup> glucan $\beta$ -Glucosidase 60 IU g <sup>-1</sup> glucan	10% WIS	34	85	Bertilsson et al. [17]
	<i>S. cerevisiae</i> , SyBE005	15 FPU g <sup>-1</sup> dry matter	25% dry matter of pretreated corn stover	34	47.2 g/L	Zhu et al. [18]
	<i>S. cerevisiae</i> IPE003	-	20% steam exploded corn stover	30	75.3% (60.8 g/L)	Liu et al. [19]
	<i>K. marxianus</i> DBKKU Y-102	-	250 g/L sugars	37	92	Charoensopharat et al. [20]
CBP	<i>Trichoderma reesei</i> Rut C30, <i>S. cerevisiae</i> and <i>Scheffersomyces stipitis</i>	-	17.5 g/L Avicel	28	67	Brethauer and Studer [21]
	<i>Scheffersomyces shehatae</i> JCM 18690	-	10% starch liquid	30	9.2 g/L	Tanimura et al. [22]
	<i>K. marxianus</i> Y179	22.9 U/mL inulinase	227 g/L inulin	30	98 g/L	Gao et al. [23]

resulting enzymatic hydrolysate is used to produce biofuel by the action of fermenting microorganisms. Both processes are performed separately because of the different temperature optima of hydrolytic enzymes (approximately 50°C) and fermentation (30–35°C). Currently, the majority of LC ethanol is produced by this process (Fig. 1).

### 3.2. SSF process

In this method, enzymatic saccharification of the pretreated biomass and fermentation of the enzymatic hydrolysate are performed in the same vessel. This method overcomes the limitation of feedback inhibition of cellulases by glucose, which is a limiting factor in SHF. Thus, SSF (Fig. 2) improves both the efficiency of enzymatic saccharification and the ethanol yield. Ghosh et al. [24] reported an increase in the hydrolysis rate by 13–30% with SSF rather than with

normal enzymatic saccharification. Ohgren et al. [25] reported a 13% higher LC ethanol yield from SSF than from the SHF process. This process mainly relies on cold-adaptive hydrolytic enzymes and thermophilic yeast. It is carried out at an ambient temperature of approximately 40°C in a single vessel.

### 3.3. SSCF process

In this process, saccharification is performed simultaneously with the co-fermentation of hexose and pentose sugars. Traditionally, LC ethanol was produced using industrially important microorganisms such as *Saccharomyces cerevisiae*. However, as the organism is incapable of fermenting pentose sugars, the substrate is not completely utilized. Therefore, in the SSCF process, organisms capable of fermenting both pentose and hexose sugars are crucial. SSCF can be

**Table 2**

Various processes for LC ethanol production along with advantages and disadvantages.

Process	Advantages	Disadvantages
SHF	<ul style="list-style-type: none"> <li>Both hydrolysis and fermentation are carried out at optimum temperatures separately</li> </ul>	<ul style="list-style-type: none"> <li>High cost requirement</li> <li>Time-consuming process as hydrolysis and fermentation are carried out separately</li> </ul>
SSF	<ul style="list-style-type: none"> <li>Reduction of cooling cost as no chiller unit is required</li> <li>Improved hydrolysis efficiency</li> <li>Reduced contamination risk</li> <li>Continuous evaporation of ethanol from culture media under reduced pressure</li> <li>Suitability for use in tropical regions</li> </ul>	<ul style="list-style-type: none"> <li>Hydrolytic enzymes are subjected to end-product inhibition</li> <li>Temperature optima of hydrolytic enzymes and yeast are different</li> <li>Reduced hydrolytic efficiency of cellulases at lower temperature</li> <li>Ethanol concentration &gt;0.2 M disturbs the adsorption of exoglucanase on cellulose and lowers the hydrolytic efficiency</li> </ul>
SSCF	<ul style="list-style-type: none"> <li>Complete utilization of substrate</li> <li>Reduced capital cost</li> <li>Higher bioethanol productivity</li> <li>Continuous removal of end products of saccharification resolve the problem of feedback inhibition</li> </ul>	<ul style="list-style-type: none"> <li>Presence of cellulase enzyme cocktails in same vessel affects yeast growth</li> <li>Difficulty in recycling of fermenting microorganism as it is mixed with the biomass</li> <li>Xylose utilization requires aerobic conditions, which inhibits glucose fermentation</li> <li>Higher affinity of glucose to transporters hinders uptake of xylose</li> </ul>
CBP	<ul style="list-style-type: none"> <li>Reduction in capital investment</li> <li>Elimination of utilities associated with enzyme production</li> <li>Single vessel for saccharification and fermentation reduces operational complexities</li> <li>Simplification of operation</li> <li>Reduction of contamination risk by reducing glucose concentration produced and ethanol produced</li> </ul>	<ul style="list-style-type: none"> <li>High loading rates affects mixing operation</li> <li>Development of new efficient microorganism capable of coproducing hydrolytic enzymes and fermentation is very difficult</li> </ul>
SSFF	<ul style="list-style-type: none"> <li>Improvement of hydrolysis efficiency by preventing product inhibition of cellulase</li> <li>Effective in enhancing cell performance</li> <li>Facilitates complete utilization of biomass</li> <li>Both biomass hydrolysis and fermentation are carried out at their optimum conditions</li> </ul>	<ul style="list-style-type: none"> <li>Clogging of membrane filters with high substrate loading is the main challenge</li> </ul>

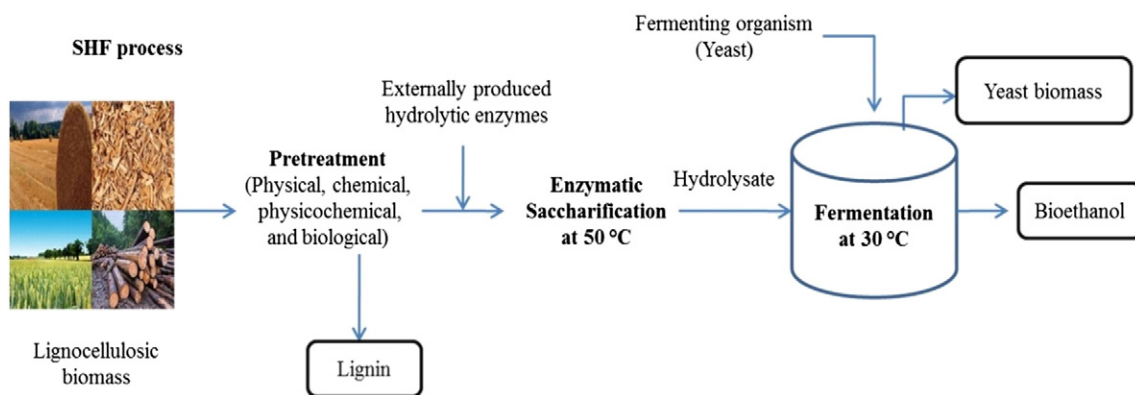


Fig. 1. The process flow of separate hydrolysis and fermentation (SHF).

operated at high content of water-insoluble solid (WIS) with fed-batch fermentation, which simplifies mixing and higher ethanol yield. It also helps in maintaining the glucose concentration at low levels, thus allowing efficient co-fermentation of glucose and xylose [26].

#### 3.4. CBP process

CBP is an integrated process with enzyme production, biomass hydrolysis, and fermentation being performed in a single step (Fig. 3). It is a promising, economical approach for producing LC ethanol, as fewer utilities are required compared to SHF and SSF. A highly engineered microbial strain that can produce sufficient hydrolytic enzymes with higher fermentation capacity is required for further development of the CBP process.

#### 3.5. SSFF process

It is an integrated process wherein a membrane filtration chamber is placed between the saccharification and fermentation chambers. Most of the engineered yeast strains have a lower affinity to xylose than to glucose; thus, xylose utilization begins after depletion of glucose from the medium. SSFF (Fig. 4) overcomes the limitations of both SHF and SSF; it also allows the separate use of both hydrolytic enzymes and the fermenting microorganism at their optimum conditions. In this process, the pretreated biomass slurry is exposed to hydrolytic enzymes. Then the mixture of pretreated biomass and enzymes is pumped through a cross-flow membrane. From this membrane, the sugar-rich filtrate is pumped to the fermentation reactor; the retentate and fermentate are recirculated to the saccharification chamber [27]. The fermented liquid is pumped back to the hydrolysis reactor to maintain the balance of volume in both reactors. Yeast

cultures exhibiting flocculating behavior are retained as they settle in the fermentation reactor [27].

#### 4. Screening of yeast strains suitable for fermentation at high temperatures

At present, bioethanol production accounts for the production of billions of liters of ethanol per annum from corn (USA) and sugarcane (Brazil). However, the use of thermotolerant fermenting yeasts can improve its efficiency by allowing fermentation to occur at temperatures above 40 °C using SSF technology. During the past few decades, many studies have reported the screening of thermotolerant yeast strains capable of fermentation at high temperatures, which are deemed suitable for bioethanol production by SSF (Table 3).

Studies have also shown that stress conditions can induce tolerance to high temperatures in *S. cerevisiae*; however, if the adapted yeast is grown under normal/optimal conditions, this thermotolerance can be lost [28,29]. In a recent study, thermotolerant *S. cerevisiae* strains isolated by physiological adaptation (adaptive evolution) to temperatures  $\geq 40^{\circ}\text{C}$  were found to have an altered sterol composition. The modification in the sterol composition of yeast strains was considered to maintain the fluidity of the cell membrane at high temperatures, resulting in increased thermotolerance [36]. Suutari et al. [28] found a negative correlation between fermentation ability and temperature increase. This correlation was attributed to changes in the membrane fluidity of *S. cerevisiae* at high temperatures. Therefore, experiments for isolating *S. cerevisiae* mutants that may be able to resist the changes in sterol composition against high-temperature stress are needed. These experiments can involve the addition of inhibitors of sterol metabolism or compounds that can alter the composition of the cytoplasmic membrane, ultimately stabilizing the membrane at high temperatures.

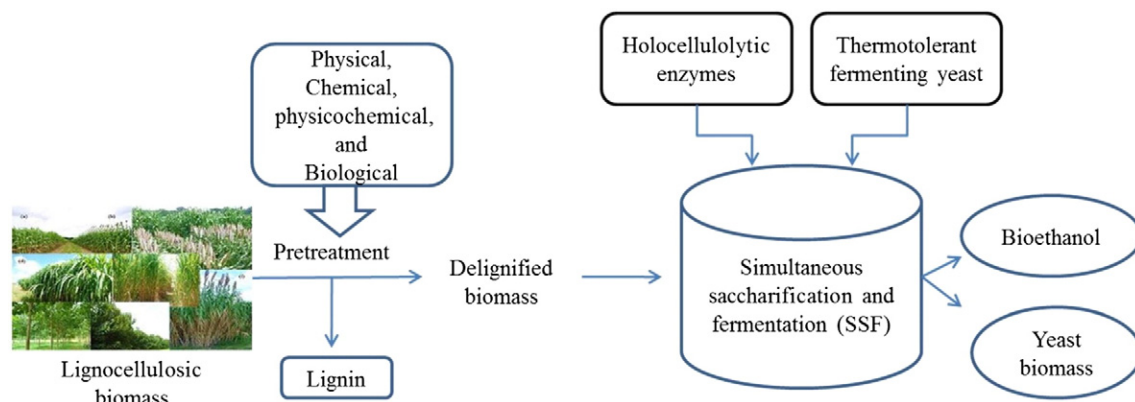


Fig. 2. Bioethanol production by simultaneous saccharification and fermentation (SSF) process.

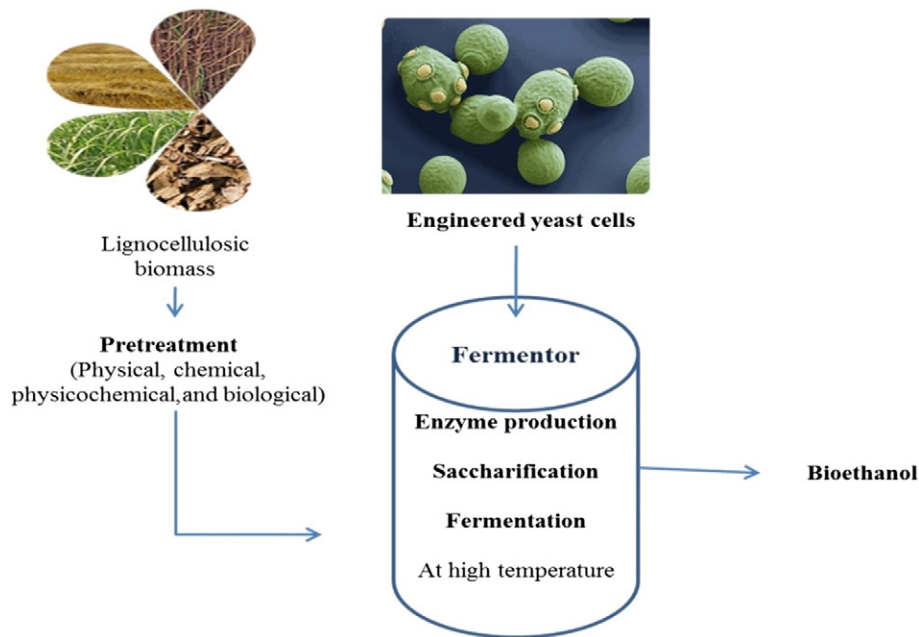


Fig. 3. Schematic representation of different processes involved in CBP for direct bioethanol production from lignocellulosic biomass.

Another approach includes screening of thermotolerant fermenting yeast strains, which are resistant to the glucose analogue 2-deoxy-D-glucose. These *S. cerevisiae* mutants show improved fermentation ability at elevated temperatures [37]. This increase in fermentation efficiency of these mutant strains was attributed to the lack of catabolite repression by glucose and improved uptake of glucose [38,39]. Similarly, a *Candida molischiana* mutant resistant to 2-deoxy-D-glucose was capable of producing ethanol at 45°C, unlike its wild-type counterparts [40].

Tropical regions are ideal sites for the isolation of thermotolerant yeast strains. *S. cerevisiae* isolates from this region can tolerate temperatures above 44°C. However, these organisms grow at a slower rate above these temperatures than in the mesophilic range of temperature, showing reduced fermentation capability [41]. Therefore, temperature is a key physical parameter that limits the performance of the organism in terms of ethanol production.

### 5. Effect of high temperature on yeast

Yeasts are mesophilic in nature. When grown beyond the optimum temperatures, yeasts show altered morphology and physiology in several ways. The growth and metabolism of yeasts at various temperatures is determined by the genetic makeup of the yeast strain, the composition of the culture medium, and other growth parameters.

The accumulation of yeast metabolites, both inside and outside the cell, may have an effect on the sensitivity of yeast to temperature. The temperature optima of yeasts vary with species. Thermotolerance is the transient ability of yeast cells to survive at higher temperatures. Thermotolerant yeast strains can survive at temperatures above 40°C. Intrinsic thermotolerance is observed in yeast cells after being subjected to a heat shock (e.g., to 50°C). However, induced thermotolerance is observed when cells are preconditioned by subjecting them to a mild heat shock (e.g., 37°C for 30 min) before a more severe heat shock. Several factors, apart from a mild heat shock, such as specific chemicals, osmotic dehydration, and low external pH, composition of culture medium, and phase of growth, are known to affect yeast thermotolerance [29]. With respect to pH changes, yeast thermotolerance increases to a maximum when the external pH decreases to 4.0. Furthermore, evidence points to the role of alterations in intracellular pH in stimulating thermotolerance in *S. cerevisiae* [42].

The growth of *S. cerevisiae* ScY and ScY01 at elevated temperatures suppressed the expression of several proteins involved in various metabolic pathways such as central carbon metabolism (CCM), lipid metabolism, amino acid metabolism, and vitamin and cofactor metabolism. Growth at elevated temperature also affects protein transport and vesicle organization. By contrast, sudden heat shock was found to increase the expression of many proteins involved in

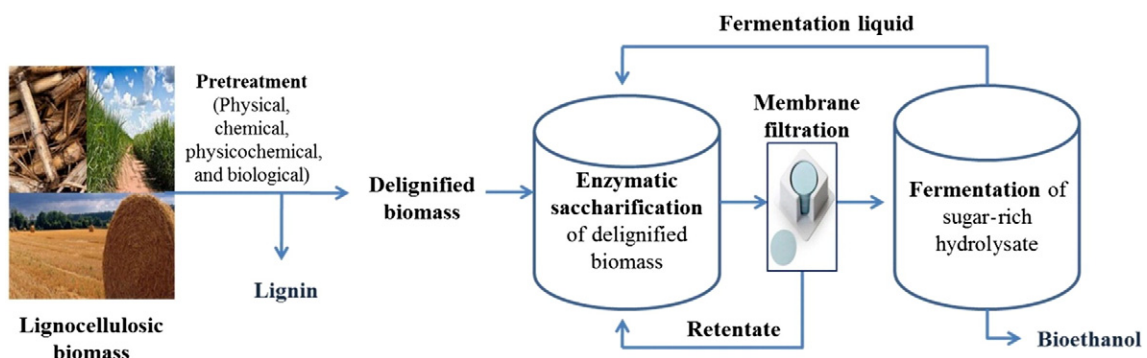


Fig. 4. Bioethanol production from lignocellulosic feedstock using simultaneous saccharification, filtration, and fermentation (SSFF).

**Table 3**  
Ethanol production from biomass using thermotolerant yeasts.

Organism	Substrate	T (°C)	Ethanol yield (g/L)	% Theoretical yield	Reference
<i>S. cerevisiae</i> ZM1-5	Sugarcane bagasse	40	18.79	82.35	Huang et al. [30]
<i>K. marxianus</i> DBKKU-Y102	Jerusalem artichoke	40	97.46	92	Charoensoparat et al. [20]
<i>K. marxianus</i> TISTR 5925	Palm sap	40	45.4	92.2	Murata et al. [12]
<i>Blastobotrys adenivorans</i> RCKP 2012	Sugarcane bagasse	50	14.05	46.87	Antil et al. [13]
<i>Pichia kudriavzevii</i> HOP-1	Rice straw	45	24.25	82	Oberoi et al. [31]
<i>K. marxianus</i> OT-1	Jerusalem artichoke	40	73.6	90	Hu et al. [32]
<i>S. cerevisiae</i> JZ1C	Jerusalem artichoke	40	65.2	79.7	Hu et al. [32]
<i>S. cerevisiae</i> TJ 14	161 g/L paper sludge material	42	40	74	Prasetyo et al. [33]
<i>K. marxianus</i> IMB3	Kanlow switchgrass	45	22.5	86	Pessani et al. [34]
<i>S. cerevisiae</i> D <sub>5</sub> A	Switchgrass	37	21.9	92	Faga et al. [35]

carbohydrate metabolism, lipid metabolism, protein folding and degradation, and oxidative stress response. In particular, specific proteins such as cytochrome b2, glycogen phosphorylase, long-chain fatty acid–coenzyme A (CoA) ligase 1, (DL)-glycerol-3-phosphatase, catalase T, and transaminated amino acid decarboxylase were downregulated in both ScY and ScY01 during their thermotolerant response and were increasingly produced in the heat-shock response [43].

CCM has been implicated in modulating yeast survival during lethal heat stress, although specific mechanisms of central metabolic genes in regulating thermosensitivity remain unknown [44]. In thermotolerant responses, Shui et al. [43] observed variation in the expression of proteins involved in CCM, such as upregulation of glycolytic enzymes and downregulation of enzymes involved in the tricarboxylic acid (TCA) cycle, glycogen and glycerol biosynthesis, pentose phosphate pathway, and components of the electron transport chain such as cytochrome b/c subunits and ATP synthases.

Yeast cells respond quickly at the molecular level when exposed to a sudden increase in temperature. This is termed as the heat-shock response. This regulatory phenomenon occurs in all living cells. Sublethal/mild heat shock treatment leads to the induction and expression of genes responsible for the synthesis of heat-shock proteins (HSPs). As *S. cerevisiae* is mesophilic in nature, it shows poor fermentation efficiency at elevated temperatures (>35°C). This is due to the increased fluidity of the cytoplasmic membranes, to which the yeast cells respond by modifying their fatty acid composition [28]. However, temperatures ≥34°C have an adverse impact on yeast cell viability and growth. Thermotolerance in *S. cerevisiae* can be induced by short-term exposure to nonlethal stress conditions including high osmolarity, low pH, high ethanol concentrations, and suboptimal temperatures (≥37°C). The induced thermotolerance is nonheritable. It can be attributed to the induction of various cellular responses such as synthesis of HSPs and trehalose, which in turn can arrest the cell cycle at the G1 phase, and reduce the activity of cyclic adenosine 3',5'-monophosphate–protein kinase (cAMP–PK) associated with low glycolytic fluxes [29,45]. Thus, the physiological adaptation of yeast is not a suitable approach for ethanol production. Multiple cell changes caused by high temperature will ultimately affect the structure and function of proteins, generate abnormal proteins, and result in growth inhibition or cell death. These degraded and denatured proteins are mainly degraded by the proteasome pathway as a defense mechanism to ensure cell survival [46]. Ubiquitination is the primary signal used to target cellular proteins and destroy them via the action of 26S proteasomes. Ubiquitin is induced by various kinds of stresses that necessitate more extensive protein turnover [47]. It is also an important non-proteolytic signal that regulates the function of proteins by nondegradative mechanisms, including the modulation of protein–protein interactions in various biological systems [48]. High-temperature tolerance is mostly controlled by the activation and regulation of specific stress-related genes. These genes play a key role in the synthesis of specific compounds that protect the organism from high-temperature stress [49]. After a sublethal heat shock,

apart from HSP synthesis, yeast cells begin to accumulate other protective compounds (e.g., trehalose) along with selected enzymes (mitochondrial superoxide dismutase and catalase). Trehalose is considered a thermoprotectant, as it helps in stabilizing the cytoplasmic membrane and cellular proteins [50].

## 6. Role of thermotolerant yeast in SSF

Commercially available cellulases and hemicellulases carry out hydrolysis efficiently at temperatures in the range 45–50°C. Further, most fermenting microorganisms used in the industry have temperature optima around 30–35°C. Thus, the process needs to be carried out separately, termed as SHF, which is an expensive and energy-intensive process. Thus, identifying thermotolerant yeasts can help in combining these processes, ultimately leading to SSF.

In a study by Shahsavarani et al. [51], a Htg<sup>+</sup> (high-temperature growth phenotype) strain showed confluent growth at higher temperatures (41°C) as well as resistance to heat-shock, ethanol, osmotic, oxidative, and DNA damage stresses. *HTG 6*, one of six genes encoding for the thermotolerant phenotype, was identified to be the *RSP5* gene, which encodes for ubiquitin ligase.

Some thermotolerant and ethanol-producing yeast strains have been isolated and modified for producing ethanol from LC biomass. *Candida glabrata* is a promising candidate for developing the SSF process, as this yeast is more tolerant to both high temperature and high acid concentration, along with superior ethanol-producing ability [52]. *Kluyveromyces marxianus* is also promising in the production of ethanol at higher temperatures. Many strains of *K. marxianus* grow well at temperatures as high as 45–52°C and can efficiently produce ethanol at temperatures ranging from 38 to 45°C [53,54,55]. Moreover, *K. marxianus* offers additional advantages such as a high growth rate and the ability to utilize a wide range of sugar substrates (e.g., galactose, arabinose, xylose, and mannose) at elevated temperatures [55,56,57]. Because of these advantages, *K. marxianus* has been used for bioethanol production with various substrates such as corn silage juice, sugarcane juice, whey powder, and molasses [58,59,60,61]. Steam-pretreated LC material (eucalyptus, poplar, bagasse, sweet sorghum, mustard, and wheat straw) was used to produce LC ethanol via the SSF process using *K. marxianus* [62]. The SSF process was carried out at 42°C with 100 g/L of the substrate and commercial cellulase @ 15 FPU/g of the substrate. After 72–82 h of fermentation, 16–19 g/L of ethanol was produced from the LC materials. The ethanol yield was 50–72% of the theoretical yield based on the glucose available in the pretreated materials. The *K. marxianus* IMB strains identified by Banat et al. [54] showed favorable SSF results at temperatures ranging from 40 to 50°C [35,63,64].

Some of the promising thermotolerant *S. cerevisiae* strains have been isolated from tropical regions [41,65]. The *S. cerevisiae* TJ14 strain has high-temperature (41°C) growth optima. It was found to produce 40 g/L of ethanol from 161 g/L of paper sludge organic material containing 66% (w/w) glucan via an SSF process at 42°C using a cellulase produced by the filamentous fungus *Acremonium cellulolyticus*

[33]. Based on classical genetic analysis of thermotolerant *S. cerevisiae* strains, it was also found that RSP5, the gene encoding for an essential E3 ubiquitin ligase, and CDC19, the gene encoding for pyruvate kinase, contribute to the high-temperature growth phenotype [51,66]. Various anaerobic bacterial species such as *Thermoanaerobacter ethanolicus*, *Thermoanaerobacterium saccharolyticum*, and *Clostridium thermotherum* have been used to ferment hexose and pentose sugars to produce ethanol [67,68,69]. However, in practice, it is difficult to maintain anaerobic conditions in large-scale fermentation, which restricts the use of thermophilic anaerobes.

In their study, Banat et al. [54] used *K. marxianus* IMB3 for the industrial-scale fermentation of molasses. They did not use a cooling system, with the fermentation temperature being increased up to 42°C. They concluded that 60–72 g/L (0.51 g/g glucose) ethanol may be produced, similar to the yield obtained regularly from *S. cerevisiae* in distilleries at lower temperatures. Pessani et al. [34] carried out fermentation of pretreated switchgrass (*Panicum virgatum*) using *K. marxianus* IMB3 at different temperatures, with an ethanol yield of 22.5 g/L, 12% solid, and enzyme loading (0.7 mL/g) of Accelerase 1500 at 45°C, achieving 86% theoretical yield.

## 7. Methods for developing yeast strains suitable for SSF

### 7.1. Site-directed mutagenesis

Using site-directed mutagenesis (SDM), also known as oligonucleotide-directed mutagenesis or site-specific mutagenesis, specific desired changes to the DNA sequence of a particular gene can be effected, resulting in altered gene products. SDM is key to investigating the structure and biological function of DNA, RNA, and proteins and also to protein engineering.

A DNA primer with the desired mutation, complementary to the genomic DNA around the mutation site, is needed to effect the desired changes in the genomic DNA sequence. The desired mutation

may be a point mutation (single base change), multiple base changes, insertion, or deletion. The single-strand primer is then extended via a DNA polymerase, which copies the rest of the gene. The copied gene contains the desired mutation. Then the gene with desired mutation is introduced into the target host via a suitable vector and is then cloned. Finally, the mutants are screened and selected by DNA sequencing to check for the presence of the gene of interest.

*Hansenula polymorpha*, a methylotrophic yeast, was subject to mutation with SDM to produce ethanol from cellobiose, glucose, and xylose at elevated temperatures [70]. Dmytruk et al. [71] improved the xylose-utilizing ability of a *H. polymorpha* strain by site-specific mutagenesis of the endogenous xylose reductase gene. The recombinant strain showed 7.3-fold higher ethanol productivity at 48°C than the wild-type strain did, thus making it a suitable candidate for use in SSF.

### 7.2. Genome shuffling approach

Zhang and coworkers first introduced this method in 2002 as a mean of improving tylosin production by *Streptomyces fradiae* [72]. DNA shuffling is used for in vitro homologous recombination of pools of selected mutant genes by random fragmentation and PCR reassembly. Similarly, genome shuffling improves the strain by introducing beneficial mutations in a directed evolution experiment. This method is used to increase the size of the DNA library. It involves recombination between genomic DNA of different strains or species with different mutations. This technique combines the advantages of multiparental crossing allowed by DNA shuffling with the recombination of whole genomes normally associated with conventional breeding, or by protoplast fusion, which enhances the recombination. In addition, genome shuffling can accelerate directed evolution by facilitating recombination between the members of a diversely selected population [72,73]. The procedure of genome shuffling (Fig. 5) consists of the following steps: construction of parental library, protoplast fusion, and selection of the desired phenotype.

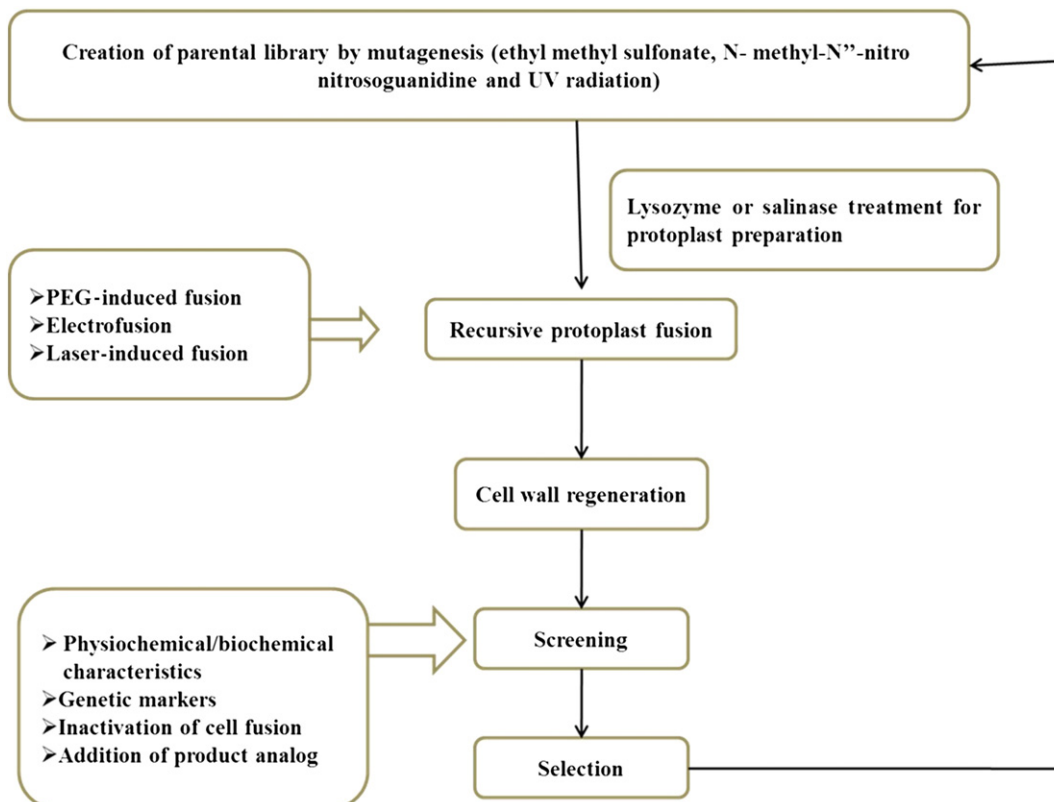


Fig. 5. Schematic representation of genome shuffling.

To construct a parental library, the initial strain is engineered via mutagenesis to generate more genotypes. Then these cells are suspended in a buffer containing lysozyme [74,75] or salinase [73]. Following this, the protoplasts obtained are aggregated by centrifugation. Further, an equal number of protoplasts from the mutants are mixed, divided into two equal parts, and inactivated by incubation at high temperature (50–60°C) or by ultraviolet (UV) irradiation [76]. The killed protoplasts are grouped together and fused in a system containing 35% polyethylene glycol and 0.1% calcium chloride at 35°C for 40 min. Then, the fused protoplasts are centrifuged; washed twice; and resuspended in 10 mL buffer, serially diluted, and regenerated. The strains from the regenerated protoplast are pooled and considered as the strain library for the second round. The same process is repeated several times. In the final step, screening is performed for the selection of the desired phenotypes. This technique has been used to enhance the product yield, the strain tolerance, and substrate uptake. The thermotolerance, ethanol tolerance, and ethanol productivity of *S. cerevisiae* F-34 were enhanced with the genome shuffling approach by a combination of protoplast fusion and UV irradiation. The strain F-34 was capable of growing at 55°C and utilizing complete sugars at 20% concentration, resulting in ethanol production (9.95% w/v) and ethanol tolerance of up to 25% v/v [77].

### 7.3. Mutagenesis

Industrially important *S. cerevisiae* is a good producer of ethanol. It shows higher tolerance to ethanol, but it lacks thermotolerance. Therefore, mutation screening for thermotolerance was performed with a proofreading-deficient DNA polymerase or UV irradiation. A *S. cerevisiae* mutant capable of growing at temperatures up to 40–42°C was selected [78,79]. A respiratory mutant of *C. glabrata* yielded 17.0 g/L of ethanol from 50 g/L of Avicel/microcrystalline cellulose at 42°C under aerobic conditions in the presence of sufficient cellulase [80].

### 7.4. Metabolic engineering

Metabolic engineering uses tools of genetic engineering to modify the metabolism of an organism. It may involve optimizing existing biochemical pathways or introducing pathway components, most commonly in bacteria, yeasts, or plants. The main aim is to produce a high yield of specific metabolites for medicine or biotechnology. Insights into yeast physiology under various stress conditions (osmotic stress, low pH, high temperature, high ethanol concentration stress, etc.) during fermentation can help guide further improvements in large-scale ethanol production by engineering stress-tolerant traits.

Auxotrophic strains are considered important platforms for both fundamental and applied research in industrial biotechnology, with the culture conditions promoting selective pressure on recombinant cells [81]. Under uracil-limiting conditions, uracil-auxotrophic strains of *S. cerevisiae* CEN.PK113-5D dissimilate the carbon source mainly into ethanol and acetate by respiratory fermentative metabolism. Under these conditions, the yeast strains also show increased specific rates of glucose, sucrose, and CO<sub>2</sub> production as well as increased O<sub>2</sub> consumption [82]. The uracil auxotrophy in the yeast is introduced by a Ty insertion mutation within the coding region of the URA3 gene. The URA3 gene in the yeast encodes for orotidine-5-phosphatase decarboxylase (ODCase), an enzyme involved in the de novo synthesis of pyrimidine nucleotides. This enzyme is responsible for the decarboxylation of orotidine-5'-phosphate (OMP) to uridine-5'-phosphate (UMP). Thus, *ura3-52* mutation inhibited the ability of the cells to synthesize UMP [83]. Therefore, uracil auxotrophy produced by Ty insertion in the URA3 gene favors ethanol production from the carbon source under uracil-limiting conditions. These cells must utilize the uracil present in the medium via the pyrimidine salvage pathway.

### 7.5. Cell encapsulation

Cell encapsulation is similar to enzyme immobilization, in that the cell is attached to a solid support such as calcium alginate, activated polyvinyl alcohol (PVA), or activated polyethylene imine (PEI). The process of cell encapsulation is presented in Fig. 6. Cell immobilization confers many advantages to fermentation such as reuse of the fermenting microorganism, high cell concentrations, easier product recovery, increased substrate uptake, lesser chances of contamination, faster sedimentation of non-flocculating cells after completion of fermentation, greater tolerance against inhibitors and high temperature [27]. Cell encapsulation helps ferment toxic LC hydrolysates and improves cell stress tolerance.

After being immobilized, yeast cells remain in close contact and form aggregates. Further, the cells grown in a small space show modified growth pattern and metabolism. In several cases, the resultant yeast community is increasingly protected from the harsh and inhibiting conditions [84]. On analyzing the membrane composition of immobilized cells, an increase in fatty acid, phospholipids, and sterol content was observed, which confers increased protection against high ethanol stress [85].

Ylivero et al. [84] reported that encapsulated *S. cerevisiae* CBS8066 (a non-thermotolerant yeast strain) successfully fermented 30 g/L of glucose with high ethanol yield in five consecutive batches of 12-h duration at 42°C, compared to freely suspended yeast, which was completely inactivated after the third batch.

### 7.6. Physiological adaptation or evolutionary engineering

This method is based on natural means of engineering: variation and selection. Here, genetic diversity is created by mutagenesis and recombination. Following this, large populations are allowed

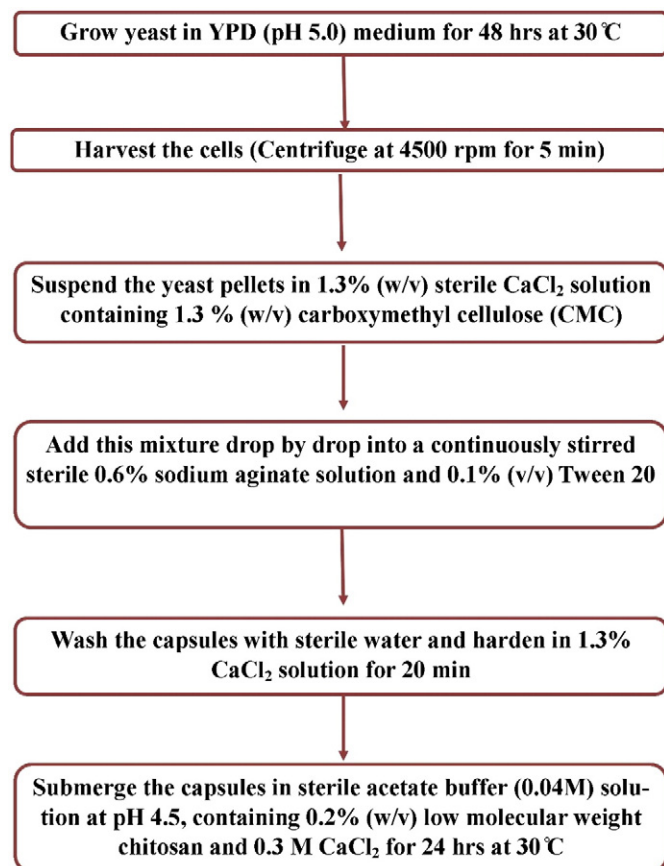


Fig. 6. Procedure for encapsulation of yeast cells for SSF.



to continuously evolve under selection pressures such as high temperature and sugar/salt concentration over several generations, relying on the cell's inherent ability to introduce adaptive mutations [86]. Thermotolerance can be developed by exposing yeast strains to a gradual rise in temperature (for example 2°C at each step) for many generations. The thermotolerance developed via this method is not permanent, and it can be lost after a few generations. Thus, physiological adaptation is not a suitable approach for developing thermotolerant yeasts.

## 8. Benefits of high-temperature fermentation using thermotolerant yeasts

Thermotolerant yeasts, which can ferment sugars at temperatures around 40°C, can be used to minimize the cost of ethanol production as discussed in the following sections.

### 8.1. Cooling costs

The production of bioethanol at higher temperatures has been increasingly studied due to several advantages such as decrease in cooling costs, constant evaporation of ethanol from the culture broth under reduced pressure, lower chances of contamination, applicability of process in tropical regions, and the increased efficiency [53,87,88]. Abdel-Banat et al. [89] reported that a 5°C increase in fermentation temperature can significantly reduce the overall cost of ethanol production from starchy material using a thermostable  $\alpha$ -amylase, thereby reducing the cooling energy. The continuous evaporation of ethanol from the fermentation broth during fermentation helps maintain the ethanol concentration at levels nontoxic to the fermenting microorganism, thereby simplifying subsequent distillation. These researchers produced ethanol from starch by supplementing glucoamylase (from *Bacillus licheniformis*) with yeast cells. They calculated a net increase in benefits associated with a 5°C increase in fermentation temperature of around US\$30,000 per annum for a 30,000-kL ethanol plant.

### 8.2. Cost reduction at the SSF stage

SSF is preferred over SHF, because it reduces the need for separate fermenters, in turn reducing the overall cost of ethanol production. The combination of saccharification and fermentation simplifies the overall process of ethanol production. Preventing feedback inhibition of cellulases by the addition of glucose helps increase the saccharification efficiency and ethanol yield. However, the major disadvantage of the SSF process compared with the SHF process is the reduced efficiency of saccharification at lower temperatures, at which yeast fermentation is carried out. Therefore, thermotolerant fermenting microbial strains capable of producing significant amounts of ethanol at high temperatures are preferred for saccharification. They are also essential for improving the efficiency of SSF. Thermotolerant yeast strains of the genera *Saccharomyces*, *Kluyveromyces*, and *Fabospora*, which can produce more than 5% (w/v) ethanol at elevated temperatures (>40°C), have been identified in several studies [12,32,34,35,90,91].

## 9. Limitations associated with high-temperature fermentation

During bioethanol production in traditional systems, yeast cells are subject to various stresses such as high sugar and ethanol concentration, low nutrient concentration, and pH change [92,93]. In addition, high-temperature stress leads to other metabolic problems during ethanol fermentation, resulting in low alcohol yield [82]. Higher temperatures can cause cell damage in yeasts in various ways, with the most serious effects being membrane disruption and protein denaturation and aggregation [94]. Growth at high temperature leads

to the partial or total alteration of the native secondary and/or tertiary structure of nucleic acids and proteins due to the breakage of H bonds and hydrophobic interactions [50]. Yeast cells are not capable of regulating their internal temperature; therefore, the cell viability rapidly decreases beyond the optimal temperature.

## 10. Conclusions and future perspectives

LC biomass has great potential for biofuel production. In particular, second-generation bioethanol can contribute to a cleaner environment and a carbon-neutral cycle. Thermotolerant and ethanologenic yeast strains can be used for bioethanol production by SSF at elevated temperatures, as they reduce the costs of the overall process. Thermotolerant yeasts can be developed by mutation, genetic engineering, metabolic engineering, and physiological adaptation. Fermentation at high temperatures with thermotolerant yeasts and saccharification simultaneously has several advantages such as reduced cooling cost, reduced need for utilities, higher saccharification efficiency, and no feedback inhibition of cellulolytic enzymes. Compared with commercial enzymes, cold-active cellulases and hemicellulases from organisms typically inhabiting temperate regions hydrolyze the biomass at low temperature but with lower efficiency. Therefore, thermotolerant yeast and cold-active hydrolytic enzymes must be developed for a more cost-effective SSF process.

Traditional methods are limited by various technological gaps. Thus, modern methods of genetic engineering such as SDM or genome shuffling along with high-throughput screening techniques can be used to enhance improved yeast strains. These methods can also be used to enhance the expression of hydrolytic enzymes to suit the SSF process. Functional genomics together with metabolic engineering can be used to develop robust yeast strains capable of fully utilizing the sugar component of LC biomass. However, construction of recombinant strains has been limited to a few species such as *K. marxianus* and *Pichia kudriavzevii* because effective genetic tools are lacking. Comparing the metabolic profiles of thermotolerant yeast and well-established mesophilic *S. cerevisiae* may further elucidate the thermotolerance mechanism of yeast. A combination of cold-active cellulolytic enzymes and thermotolerant yeasts can overcome the problem of different temperature optima in the SSF process. However, further research into the sugar uptake mechanism, effects of inhibitors on yeast growth, and metabolic engineering for generating co-fermenting yeasts is needed.

## Conflict of interest

The authors declare that they have no competing interests.

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