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Study on the cellulase-producing and thermotolerant yeast strains and the cellulosic ethanol fermentation

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Abstract

Cellulosic ethanol is an important and sustainable biofuel to lessen petroleum dependence and food crop usage. Currently, the high cost of lignocellulosic processing is the major technical barrier in its commercialization. Reduction of cellulase usage and high temperature fermentation are among the practical solutions on cost reduction of cellulosic ethanol production.

In the first part of this thesis, a β-glucosidase enzyme producing yeast strain was used in cellulosic ethanol fermentation for the purpose of reducing cellulase enzyme usage. Cellobiose inhibits the cellulase enzyme and reduces the hydrolysis yield of glucose, eventually affects the final ethanol production efficiency. We used a native β-glucosidase producing yeast strain *Clavipora* NRRL Y-50464 for the purpose of reducing the cellulase enzyme dosage. *Clavispora* NRRL Y-50464 was used in separate enzymatic hydrolysis and fermentation (SHF), as well as simultaneous saccharification and fermentation (SSF) operations. The cellulase dosage was reduced to 5 mg/g of cellulose in comparison with the conventional *Saccharomyces cerevisiae* yeast strain. The ethanol titer of 38.1 g/L and the conversion yield of 55.5% were obtained at 25% solids loading (w/w) in SSF without the addition of an extra β-glucosidase. This study showed the potential of *Clavispora* NRRL Y-50464 for reduction of cellulase enzyme used and lower-cost cellulosic ethanol production from lignocellulosic biomass.

In the second part of the thesis, a thermotolerant and xylose utilizing strain *Saccharomyces cerevisae* Z100 from long term adaptive evolution was applied to cellulosic ethanol fermentation under very high fermentation temperature. The conventional fermentation requires high energy input and water usage to maintain the fermentation temperature around 30 ºC. This thesis used a newly adapted thermotolerant and xylose utilizing strain *S. cerevisiae* Z100 for simultaneous scarification and co-fermentation (SSCF) at very high temperature up to 52 ºC under high solids loading (30%). The results showed that *S. cerevisiae* Z100 produced 80.1 g/L of ethanol with a yield of 77.7% and the productivity of 0.668 g/L/h). The present work was also with the properties of zero wastewater generation, zero cooling water utilization, and less cell death during high temperature fermentation.

The overall activities of this thesis demonstrated the potentials of fermenting strains for lower-cost cellulosic ethanol production using lignocellulosic biomass. The results would contribute to the cost of effective cellulosic ethanol production in the future.

Keywords: Cellulosic ethanol; β-glucosidase; *Clavispora* NRRL Y-50464; thermotolerant *Saccharomyces cerevisiae* Z100, simultaneous saccharification and co- fermentation (SSCF)

产纤维素酶嗜热酵母菌株的改造与纤维素乙醇发酵

摘要

纤维素乙醇是一种重要的可持续生物燃料，使用木质纤维素进行乙醇生产可以降低对石油的依赖和粮食作物的使用。目前，木质纤维素加工过程的高昂成本是阻碍纤维素乙醇商业化的主要技术屏障。降低纤维素酶用量和高温发酵是减少纤维素乙醇生产成本的有效解决方案。

本论文的第一部分，我们使用了一种产 β-葡萄糖苷酶的酵母菌株进行了纤维素乙醇发酵以降低纤维素酶的使用。纤维二糖抑制纤维素酶活性且降低葡萄糖的糖化得率，最终影响乙醇生产效率。本部分研究使用了一株天然产 β-葡萄糖苷酶的酵母菌株 *Clavipora* NRRL Y-50464 进行了分步糖化与发酵（SHF）和同步糖化与发酵（SSF）。相比于传统的不产 β-葡萄糖苷酶的菌株 *Saccharomyces cerevisiae*，其纤维素酶用量降低至 5 mg/g 纤维素。在不添加 β-葡萄糖苷酶下进行了固含量为 25%（w/w）的 SSF，乙醇发酵浓度为 38.1 g/L，转化率为 55.5 %。本研究表明使用 *Clavipora* NRRL Y-50464 有望降低纤维素乙醇生产时的纤维素酶用量乃至纤维素乙醇的生产成本。

本论文的第二部分，我们使用通过长期适应性进化得到的耐高温木糖利用菌株 *Saccharomyces cerevisiae* Z100 进行了高温纤维素乙醇发酵。传统的纤维素乙醇发酵需要投入高的能量和水用量用于维持 30 ºC 左右的发酵。本论文使用了一株新的耐高温和木糖利用驯化菌株 *S. cerevisiae* Z100，在高温下（52 ºC）进行了固含量为 30% (w/w)的同步糖化与共发酵（SSCF），其中，乙醇发酵浓度、得率和产率分别为 80.1 g/L、77.7%和 0.668 g/L/h。本研究也实现了高温发酵过程中的零废水产生、零冷凝水使用以及更低的细胞死亡。

本论文证明了使用产纤维素酶和耐高温酵母菌株可以降低木质纤维素乙醇的生产成本，该结果将有助于实现纤维素乙醇的经济性生产。

Keywords: β-葡萄糖苷酶，纤维素乙醇，*Clavispora* NRRL Y-50464，驯化耐热酿酒酵母 Z100，高固含量麦秆，酿酒酵母 XH7，同步糖化发酵

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Chapter 1

General Introduction

1.1 Lignocellulosic Biomass

Lignocellulosic biomass is a promising alternative feedstock for liquid biofuels and bio-based chemicals production due to its relatively low cost, great abundance, and sustainable supply. Plant biomass consists of 40-55% cellulose, 25-50% hemicellulose and 10-40% lignin, depending on whether the source is hardwood, softwood, or grasses [1]. Cellulose is a linear structural component of a plant's cell wall consisting of a long-chain of glucose monomers linked by β (1-4) glycosidic bonds that can reach several thousand glucose units in length. The extensive hydrogen linkages among molecules lead to a crystalline and strong matrix structure. Hemicellulose has a lower molecular mass than cellulose and is composed of mainly pentose sugars (like xylose and arabinose) and hexoses (like mannose, glucose, and galactose) and may contain sugar acids (uronic acids) namely, D-glucuronic, D-galacturonic and methylgalacturonic acids. Its backbone chain is primarily composed of xylan β (1-4) linkages that include D-xylose (nearly 90%) and L-arabinose (approximately 10%). Branch frequencies vary depending on nature and the feedstock sources. The hemicelluloses of softwood are typically glucomannans sugars while hardwood hemicellulose is more frequently composed of a pentose sugar, xylans. Hemicelluloses are linked to lignin by covalent bonds. Lignins are phenolic compounds which are made by polymerization of three types of monomers (p-coumaryl, coniferyl, and synapse alcohols); that is responsible for providing rigidity to the plant cell wall and resistance against microbial attack [2]. The naturally crystalline properties of cellulose fibrils make it the enzymatic hydrolysis highly resistant to enzymatic actions, and thus cellulose non-crystalline properties are more important for efficient enzymatic hydrolysis operation. Furthermore, lignin can permanently adsorb enzymes inhibiting their action on the cellulose chains, and this process is rapid at higher temperatures and lower pH [3]. The addition of surfactants during fermentation operation [4], higher pH and certain pre-treatments have been found to reduce the enzyme adsorption onto lignin. Moreover, efficient enzymatic hydrolysis of biomass, the crystalline structure of cellulose recalcitrance such as lignin and hemicellulose fragmentation are essential to improve the accessible area of the cellulose pore size, leading to efficient accessibility of cellulase enzymes into the cellulose. The composition of various lignocellulosic biomasses available in abundant that can be effectively converted into various value-added products is elaborated in (Table 1.1). The idea of biorefinery has been introduced in this context as the utilization of biomass for biofuels and biochemical products. National Renewable Energy Laboratory (NREL), defined the biorefinery is an ability that combines lignocellulose biomass transformation operation and utilization of proper apparatus to generate biofuels, power and biochemicals from feedstock [错误!未找到引用源。]. Lignocellulose biorefinery is parallel to a petroleum refinery in terms of product formation but different in raw materials

utilization. Lignocellulosic biomass-based biorefinery products such as ethanol, glycerol, amino acids, lactic acid, and other valuable biochemicals are environmentally friendly. On the other hand, petroleum refinery products such as liquid petroleum, kerosene, diesel and gasoline are major contributors of environmental pollution. Currently the effect of this petroleum refinery products has been lowered due to the emergence of lignocellulose biomass-based productions such as biofuels and other biochemical commodity utilization [7]. Lignocellulose based biorefinery suffered by several operational problems and needs a proper, sustainable and cost-effective solutions before large scale industrial operations. In the preceding years, special devotion has been given for enhancing the lignocellulose bioconversion methods especially by improving the pretreatment methods, developing native cellulase producer, widespread biomass utilized and thermotolerant strains, designing special bioreactor apparatus etc. are some of them to increase biofuels and other value-added biochemical commodities[7][8][9][10]. However, for efficient cellulosic ethanol production the following cost-effective alternatives should be given prior attention, these are; (i) use of innate β-glycosidase enzyme produced strain for cellobiose degradation as a cost-effective alternative of ethanol production (ii) use of thermotolerant and xylose utilized strains *S. cerevisiae* Z100 for one-pot cellulosic ethanol production (iii) perform SSF at high solids content. Besides, avoiding the cellulase enzyme during seed culture and performing of evolutionary adaptation for ethanol fermenting strains, perform pretreatment and SSF at continuous mode and decrease cellulase dosage and its cost. In this thesis, efforts are put together to solve some of the above difficulties, and details are discussed in the next chapters.

In the present study, corn stover and wheat straw were used as a cost-effective carbon source for Strain *Clavipora* NRRL Y-50464 and parental *S. cerevisiae* XH7 respectively for ethanol production. The former strain has the potential to produce innate β-glucosidase for cellobiose degradation, and the later has been adapted thermotolerant strain and developed in our Laboratory .for cost-effective cellulosic ethanol production. This thesis consists of four chapters, including, a general introduction, research work, and a general conclusion. Chapter 1, It describes opportunities and challenges to lignocellulose biomass-based biorefinery for bioethanol production. The research work in chapter 2, improved cellulosic ethanol production from corn stover with a low cellulase input using a β-glucosidase-producing yeast subsequent a dry biorefining process [11], published in Bioprocess and Biosystems Engineering. It consists of an introduction to innate β-glucosidase producing strain potentials for ethanol production without extra β-glucosidase enzyme addition at high solids loading. The significance of this work is the utilization of innate β-glucosidase producing strain to reduce the cellulase enzyme addition. Without the addition of external β-glucosidase and with a lower cellulase input at 5 mg protein/g glucan during SSF operation, this practical result can reduce the enzyme cost significantly. Chapter 3 is under preparation for submission in the academic journals. This research paper describes the importance of a long term evolutionary adaptation of adapted yeast strain *S. cerevisiae* Z100 for two primary reasons:- i) To develop strain for utilizing xylose significantly as compared to the parental strain XH7 at high temperature and ii) To evaluate efficient integration of cellulase enzyme hydrolysis and fermentation for cellulosic ethanol

production potential of the adapted thermotolerant strain Z100 compared with the parental strain *S. cerevisiae* XH7. The result showed better growth of the strain and produced a significant amount of cellulosic ethanol titer, yield and productivity. In general the present practical studies have been the following, advantages i) Reduced the cellulase enzyme dosage and improved the hydrolysis operation for cellulosic ethanol production in both studies ii) It reduces cooling cost iii) it increases the cellulase activities iv) it lessens the cell death during temperature fluctuations v) reduce contamination vi) reduce energy and enzyme cost in large scale industrial lignocellulosic feedstock based cellulosic ethanol production. Furthermore, the research work is vital in terms of findings of high-temperature tolerance and xylose utilizing adapted thermotolerant strain *S. cerevisiae* Z100 towards cost-effective lignocellulosic-based ethanol production. Chapter 4, general conclusion followed by future work suggestions.

Table 1.1 Composition of various lignocellulosic biomasses [13]

Biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ash (%)	References
Sugarcane bagasse	32-48	19-24	23-32	1.5-5	[14]
Corn stalk	39-47	26-31	3-5	12-16	[14]
Rice huks	31.3	24.3	14.3	23.5	[18]
Rice straw	28-36	23-28	12-14	14-20	[14]
Wheat straw	33-38	26-32	17-19	6-8	[14]
Groundnut shell	35.7	18.7	30.2	5.9	[19]
Coconut shell	29.7	NA	44.0	0.5	[21]
Corn stover	38-40	28	7-21	3.6-7	[14]
Cotton waste	80-95	5-20	-----------	--------	[14]-[17]
Soft woods	45-50	25-35	25-35	-------	[22]-[24]
Hard woods	40-55	24-40	18-25	NA	[22]-[24]
News paper	40-55	25-40	18-30	8.8-1.8	[23]-[24]
Algae(green)	20-40	20-50	NA	NA	[14]-[17]

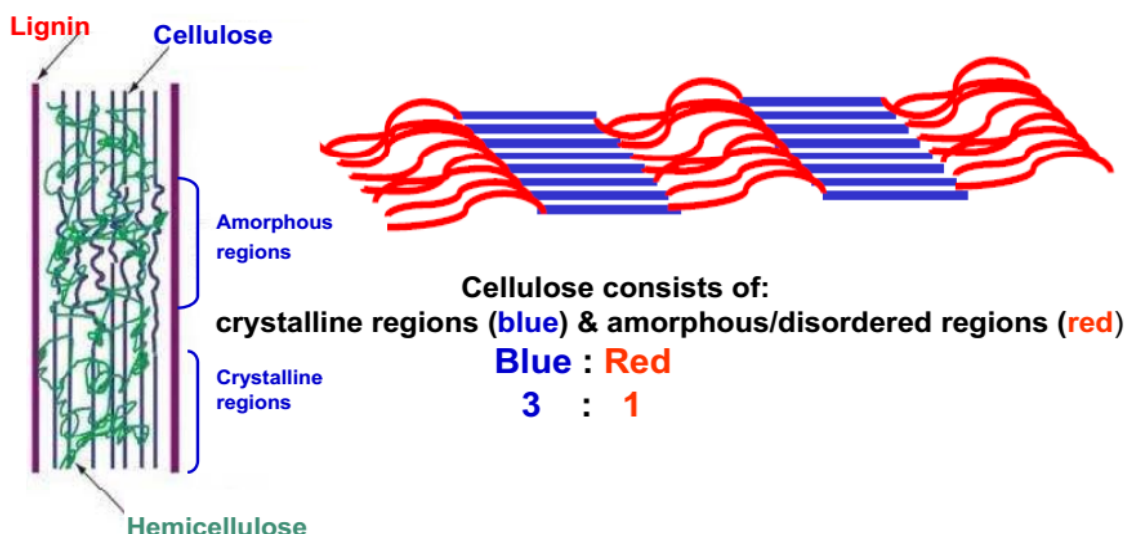


Figure 1.1 Schematic presentation for composition of lignocellulosic biomass[13]

1.2 Lignocellulose biomass for bioethanol production

Ethanol produced from lignocellulose biomass, is environmentally friendly forms of biomass energy, has the promising alternative to be a sustainable and cost-effective transportation fuel, as well as a fuel oxygenate that can replace gasoline. Previous study, [25]][27]] concluded that the energy content of ethanol was higher than the energy needed to yield ethanol[28], also estimated the total energy necessity for making ethanol from corn grain at 560 kJ MJ−1 of ethanol, showing that ethanol used as a liquid transportation fuel could lessen local intake of fossil fuels, particularly petroleum. Currently, the transportation sector all over the world is almost exclusively dependent on petroleum-based fuels. Petroleum-based fuels are responsible for 60% of world oil consumption [29]. Out of this per cent, the transportation part responsible for more than 70% of global carbon monoxide (CO) discharges and 19% of global carbon dioxide (CO_2) release [30]**错误!未找到引用源。**. The discharge of CO_2 from a gallon of gasoline is around 8 kg [31]. All over the world, there were about 806 million cars and light trucks on the road in 2007 [32]. These numbers are projected to increase to 1.3 billion by 2030, and in 2050, it would become over 2 billion vehicles [33]. This development will interrupt the constancy of ecologies and global climate and reduced the global oil reserves. The sharp rise in the price of fossil fuels, the limited nature of petroleum, increasing worries regarding energy source, environmental influence, especially related to greenhouse gas emissions, and health and safety cares are forcing to look other alternative inventive energy sources and solutions to power the world's motor vehicles. Alternative fuel, in this regard, must be practically maintainable, economically cost-effective, environmentally friendly, and easily obtainable. Today Brazil and the US are major producers of ethanol from sugarcane and corn grain respectively, which account for about 62% of world ethanol production. The major feedstock for the production of ethanol in Brazil is sugarcane, while corn grain is the main feedstock for ethanol in the USA. However, the sugarcane and corn grain feedstock used for ethanol production were not acceptable in the majority of the global community due to the large area of deforestation and competent for food, respectively. Another potential alternative resource for ethanol production is lignocellulosic biomass, which includes materials such as agricultural residues (e.g., corn stover, wheat straw, rice straw and sugarcane bagasse), herbaceous crops (e.g., alfalfa, switch-grass), forestry wastes, waste paper, and other wastes [35]. Consequently, research efforts on renewable resources such as lignocellulosic feedstock largely from agricultural feedstock such as corn stover, wheat and rice straw have become more focused on the cost-effective and environmental friendly point of view. Fuel ethanol production from lignocellulosic biomass promises cellulosic ethanol potential with some limitations. The challenging obstacles such as high production cost, high equipment requirements for fermentations, large water utilizations and cellulase enzyme cost,[36] which need to be much attention for economic and technical feasibility for bioethanol production. In general, biomass bioconversion processes includes three major steps namely (a) pretreatment of lignocellulosic biomass for removal of

hemicellulose-lignin fraction and easy accessibility to the cellulose hydrolyzing enzymes, (b) enzymatic hydrolysis of cellulose and hemicellulose occurs in the pretreated and biodetoxified biomass to get simple sugars and (c) the fermentation of the hydrolyzed sugars converted into ethanol. Biomass pretreatment mostly comprises of physical, chemical or thermal methods and in combinations.[37] (Table 1.2). During enzymatic hydrolysis, the addition of cellulose hydrolyzing enzymes like cellulases and β-glucosidases used for converting the oligosaccharides into simpler sugars like glucose, which can be easily utilized for ethanol fermentation in successive steps. Most of the cellulase combinations produced from yeast species have low β-glucosidase activity, essentially requiring supplementation of β-glucosidase for efficient hydrolyzation of cellobiose to glucose. It is well known that the production of enzymes is a cost-intensive process, and cellulolytic enzymes cost nearly 40–50% of the process expenses. Thus a small reduction in the enzymes supplementation will play a great role in improving the overall process.[38]. On the other hand, one of the essential operation options for ethanol production, when lignocellulose feedstock was used, in simultaneous saccharification and ethanol fermentation (SSF) to reduce product (sugars) inhibition on the cellulase enzymes and reduce capital cost [1]. However, the operation took place using optimal temperatures by ethanologenic microorganisms (generally below 37 ℃) and the cellulase enzymes used (50 ℃ or above) is a great challenge. Consequently, this temperature fluctuation between the cellulase hydrolysis and the fermentation caused the high cost of cellulosic ethanol production. Although several thermotolerant *S. cerevisiae* strains were tested at 40 ℃ and above, none of the strains was functional in the SSF operation at the high temperature (40 ℃ and above) and solids loading of the pretreated lignocellulosic biomass to produce high ethanol yield, which is necessary by the downstream cost-effective product purification and reduction of the distillation energy cost [41][11]. In general for efficient, cost-effective and environmentally friendly cellulosic ethanol production, one should consider the overall operational costs of the operation using lignocellulose material for ethanol production based on the following procedures: pretreatment, detoxification, enzymatic hydrolysis, fermentation, and distillation to ethanol and this operation have zero wastewater generation from pretreatment to fermentation [8][9][10][11].

1.2.1 Pretreatment of lignocellulosic biomass and effect of inhibitors

The main objective of pretreatment of lignocellulosic biomass is to remove recalcitrance nature of the cellulose by degrading lignin and hemicellulose from cellulose, then reduce cellulose crystallinity, and to increase the porosity of lignocellulose cellulose while minimizing the loss of fermentable sugars required for lignocellulosic ethanol formation. Several pretreatment plans have been developed to increase the accessibility of cellulose and increase the yield of fermentable sugars. The following are significant criteria of efficient pretreatment conditions (1) efficient reduction of the crystallinity and degree of polymerization of cellulose as well as improved in the porosity of the lignocelluloses for permitting the enzyme to enhance sugar yields during enzymatic hydrolysis, (2) avoid the chemical destruction of sugars (specifically, hexose and pentose's) as well as those derived from cellulose and hemicellulose components, (3) Reduce the formation of inhibitory products for the next fermentation

operations, (4) Transformed lignin should be recovered into valuable biochemicals, and (5) The energy utilized for ethanol production should be reduced and cost effective [42][43]. The method used for the process of pretreatment varies based on the type of feedstock due to variations in their physical features and chemical composition. Consequently, the inhibitors formed in different feedstocks also varies. The elimination of the inhibitors (such as phenolics, weak acids and furans) produced during the process using Physico-chemical and biological methods even additional to the cost of pretreatment operations [35]. Lignocellulosic biomass can be pretreated using Physico-chemical and biological processes described in (Table 1.2). Therefore, the use of an environmentally friendly pretreatment technology is mandatory to increase large scale industrial lignocellulosic ethanol yield. The adjustment of the lignocellulosic substrate is achieved through various pretreatment technologies that interrupt the cell wall structure and make it reachable to enzymes. The effect of pretreatments on lignocellulosic biomass is presented in Fig. 1.2. In addition, the formed inhibitors were released during pretreatment of lignocellulosic biomass, and their composition depends on the feedstock, pretreatment methods, and pretreatment conditions and concentration of any chemical used, pH, temperature, and residence time. The yield and rate of lignocellulose biomass to ethanol conversion was highly lowered due to the formation of cytotoxic-inhibitory compounds produced from cellulose, hemicellulose and lignin destruction and sugars drying out during the operation of the pre-treatment, which are finally discharged into the hydrolyzate together with the sugars used for fermentations. The pre-treatment phase is vital for lignocellulose biomass bioconversion into valuable biochemical. Effective delignification of the cellulose-hemicellulose-lignin complex during pretreatment allows the enzymes possible access to degrade hemicellulose and cellulose during enzymatic hydrolyzation into simple sugars (hexoses and pentose). Various pretreatment methods have been introduced, including acid/alkaline hydrolysis [44][45], steam/thermal explosion and hot water treatment [45]-[48] and ammonia fiber expansion [49]-[51] (Table 1.2). But all these methods have their own benefits and limitations. Since lignin, is one of the primary sources of these inhibitory compounds particularly inhibitors like, phenolic compounds and its kind and quantity of these inhibitors and the lignin structural qualities, degree of interaction with cellulose and hemicellulose is significantly dependent on the source of the feedstock, sources used for ethanol production [1][52][53][54]. Furthermore, the pre-treatment operation condition variables (e.g. oxygen concentration, pH of the medium, etc.) can aggravate the toxic effectors of the inhibitors [55][55]. Nevertheless, of the source or biomass preparation approaches used, during the pre-treatment and hydrolysis steps, three major inhibitory compounds are formed, namely, weak acids, furan derivatives and phenolic compounds [56]. A detailed description of these toxic compounds as follows:-

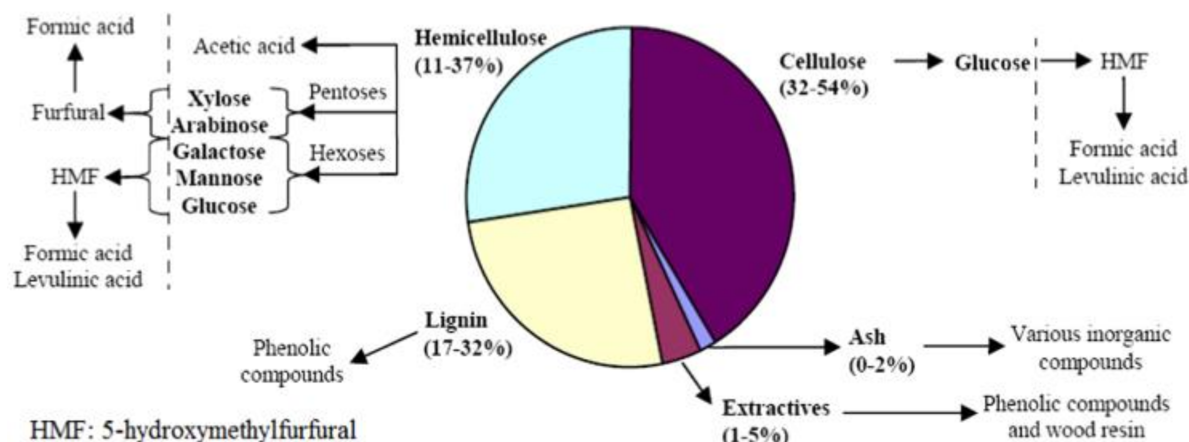


Fig. 1.2 Compositions of lignocellulosic materials and their potential hydrolysis products and formed inhibitors (Source: ncsu.edu/bioresources).

Table 1.2 The comparison of different pretreatment methods for lignocelluloses [35]

Pretreatment methods		Advantages	Disadvantages
Physical	Mechanical splintered	Reduce particle size and cellulose crystallinity	Cannot remove lignin and hemicelluloses, high energy
	Microwave	Simple operation, energy-efficient, a short time	High cost
	Ultrasonic	Improve accessibility and reactivity of cellulose	Negative to enzymatic hydrolysis
	High-energy electron radiation	Reduce cellulose polymerization degree	High cost
	High-temperature pyrolysis	Decompose cellulose rapidly	Energy consumption, low productivity
Chemical	Concentrated acid	High sugar conversion	Highly toxic and corrosive, high cost
	Dilute acid	Fast and don't need to recycle acid	High temperature and pressure, the formation of inhibitors
	Alkali pretreatment	Room temperature, destroy lignin	Less sugar degradation
	Oxidation pretreatment	Environment-friendly, remove lignin effectively	High cost,
	Organosolv pretreatment	Obtain pure lignin, cellulose, and hemicelluloses	High cost, certain effects on environment and fermentation
	Ionic liquid pretreatment	Environment-friendly, large temperature range	High cost
Physicochemical	Steam explosion	Lignin transformation, hemicelluloses solubilization, Cost-effective	High temperature and pressure
	AFEX method CO_2 explosion	increase the surface area of cellulose, no inhibitor formation	High cost, Not efficient for raw high lignin content material
	Electrical catalysis	No inhibitor formation, cost-effective, Increases surface area	High pressure, do not affect lignin and hemicelluloses
Biological	Microbes	Degrades lignin and hemicellulose Low energy consumption	Low rate of hydrolysis

First, **organic acids**, during the pretreatment and hydrolysis operations, the main organic acids (weak acids) such as lactate, succinate, formate and acetate are formed[60]. The latter, major organic acid produced, is formed from the dehydration of released sugars and decomposition of acetyl xylan, a byproduct of hemicellulose degradation (Fig. 1.2) [61][62]. While formic and levulinic acids are products of HMF breakdown. Formic acid can additionally be formed from furfural under acidic conditions at elevated temperatures. Acetate is liposoluble compound and therefore diffuses through the bacterial semi-permeable membrane and dissociates into its anionic form, then releasing protons into the cytosol [63] (Fig. 1.2). This effect causes lowering the cytosolic p^H, leading to disturbance of the trans-membrane p^H, various damaging anion-specific effects on metabolism, protein or enzyme activity/stability, and higher turgor pressure within the cell[63]. The higher p^H intracellular of ethanologenic bacteria due to the dissociation of this weak acid inside the cytoplasm has a cause of unfavorable effect on the strain cell growth and proliferation; the result does not cause to a lowering the ethanol yield [64] [64]. However, cells tend to quickly generate ATP to sustain the internal p^H and driving the strain to shift into anaerobic fermentation, consequently producing ethanol at the cost of biomass formation [64]. In general, weak acid inhibitors are the result of a reduction in cell growth and proliferation[57]. Second, **furan derivatives**, during acid pretreatment and hydrolysis of lignocellulose biomass, the furan compounds 5 - hydroxymethyl - 2 - furald-hyde (HMF) and 2 - furaldehyde are formed by dehydration of hexoses and pentose's, respectively. (Fig. 1.2) [65] The level of furans varies based on the type of feedstock and the pretreatment technique. The higher hydrophobicity of furan derivatives permits furfural and HMF to compromise membrane integrity leading to extensive membrane distraction and leakage, which finally will cause a lessening in cell duplication rate, ATP formation reduction, and consequently reduce ethanol yield. The toxicity results from the inhibition also affect glycolytic and fermentative enzymes vital to operating metabolic pathways (such as pyruvate, acetaldehyde and alcohol dehydrogenases[66], on the other hand, the effect of this inhibitors also affects protein-protein cross-linking and DNA degradation into single strands [67]] (Fig. 1.2). While the mechanism and range of cytotoxicity of lignocellulose inhibitory compounds generally differ, but they all result in gross physiological and metabolic fluctuations in the ethanogenic microorganisms, which consequently caused in lessened cell viability and fermentation efficiency. Third, **phenolic compounds,** during the degradation of lignin and dehydration of sugars in the pre-treatment and hydrolysis stages the formation of phenolic compounds were insoluble or partly soluble in the hydrolysate it include acids (ferulic acid, vanillic acids, 4-hydroxybenzoic acid and syringic acid), alcohols (guaiacol, catechol and vanillyl alcohol) and aldehydes (vanillin, syringic aldehyde and 4-hydroxylbenzaldehyde) [53] [54]. These inhibitory compounds are a significant barrier into biological membranes altering the permeability of the cytosol and lipid/protein ratio of the cell membrane, which thus escalates cell fluidity, leading to plasma membrane distraction, the degeneration of proton gradients and affecting the ability of plasma membranes to act as selective barriers [71]. This plasma membrane distraction, permits the release of proteins,

ribonucleic acid, Adenosine triphosphate, Ions, out of the cytoplasm, consequently causing to lower Adenosine triphosphate levels, weakened proton motive force and affect the overall activity of the cytoskeleton [71]. Furthermore, they initiate the formation of reactive oxygen species that cooperate with protein enzymes, which results in their denaturation and damage cytoskeleton and inhibit enzyme activity and protein functioning. In addition, it also resulted in nucleic acid mutagenesis and made automatic cell lysis (Fig. 1.2)[72]. Phenolic compounds have been informed to be more toxic than furfural and HMF[73][74]. In general inhibition mechanisms of phenolic compounds on *S. cerevisiae* and other eukaryotic microorganisms have not yet been fully clarified, mainly due to the heterogeneity of the organism and the lack of precise qualitative and quantitative analyses.

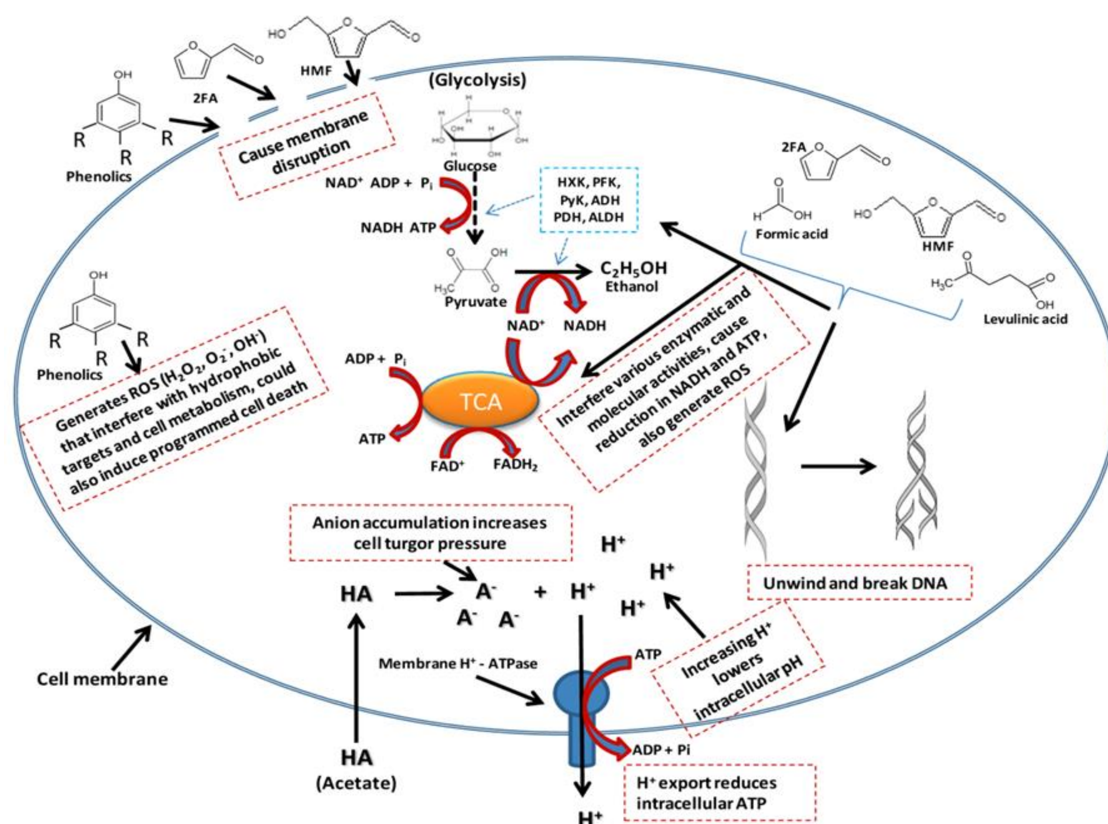


Fig. 1.2 Inhibition mechanism of lignocellulose-derived inhibitors on *Saccharomyces cerevisiae* [57]

1.2.2 Detoxification

The lignocellulose biomass after pretreatment, the hydrolysates contain some organic acids such as acetic acid, formic acid, and levulinic acid. The former formed by the hydrolysis of acetyl groups of hemicellulose and the dehydration of released sugars, the formic acid formed after degradation of furfural and HMF (5-hydroxymethylfurfural), on the other hand, levulinic acid formed after degradation of HMF [75]. These organic acids inflow into the cytoplasm observed to inhibit cell growth so that cell growth and its efficient productivity was inhibited [77][78]. These acids partially deactivate the enzymes if there concentration is so high and eventually resulted in the yeast cells death. Consequently, the toxins seriously affect the cell viability, inhibit the subsequent enzymatic hydrolysis and reduce the ethanol yield during fermentation [82][83]. Therefore, a detoxification step is mandatory to remove the toxins before subsequent enzymatic hydrolysis and fermentation. Various detoxification methods had been tried in the last decades, such as vaporization, water washing, and over-liming, etc.[84]**错误!未找到引用源。** . However, these methods resulted in many negative outcomes, including a large amount of freshwater utilization and wastewater formation, removal of the adequate lignocellulose components and fermentative sugars and poor reduction of toxins. An alternate option for avoiding toxins without causing these problems is using biological organisms the so-called biodetoxification, which beliefs microorganisms to degrade the toxins as part of their natural metabolism by secreting peroxidase or laccase enzymes into the hydrolysate [36][80][81][85]. Unlike the chemical-based detoxification, method biodetoxification has many benefits, such as zero loss of cellulose solids, greatly reduced use of water, and provide a high yield of sugars for fermentation and environmentally friendly. Currently, we used *A. resinae* ZN1 adapted to the pretreated lignocellulose materials, the strain environmentally friendly, robust growth in the presence of major inhibitors as the carbon source and preserved the cellulose components. Besides, in contrast to the previous detoxification methods, the detoxification using *A. resinae* carried out by the solid-state fermentation on the pretreated lignocellulose materials, thus directly utilizing the toxin compounds at its higher concentrations with zero wastewater generations and zero loss of major hydrolysis sugars.

1.2.3 Hydrolysis

The conversion of carbohydrate polymers of lignocellulosic biomass into simple sugars before fermentation using cellulase enzymes through a process called Hydrolysis [53]. Various methods of lignocellulosic feedstock hydrolysis, have been applied. The most commonly used methods can be ordered into two categories: chemical hydrolysis (Dilute and concentrated acid hydrolysis) and enzymatic hydrolysis. Chemical hydrolysis encompasses the accessibility of lignocellulosic materials to chemical compounds for efficient hemicellulose and lignin degradations at a specific temperature and residence time result in sugar monomers from cellulose and hemicellulose polymers[53]. The first step chemical hydrolysis, the pretreatment,

and the hydrolysis may be carried out in a single step. Acids mainly applied in chemical hydrolysis [86]错误!未找到引用源。. The structural, physical and chemical conditions of lignocellulosic biomass affect the direct hydrolysis lignocellulosic biomass. Thus, the purpose of any pretreatment operation is to make the biomass accessible to chemical and enzymatic hydrolysis for achieving a high yield of sugars for fermentation [1]. Pretreatment of lignocellulosic biomass is usually preformed before its hydrolysis and used to enhancing change of cellulosic crystallinity, its degree of polymerization, cellulose pore size improvement for hydrolytic enzymes or chemicals, to get more access to degrade cellulose-hemicelluloses and lignin complexes [87]. Therefore, the efficiency of any hydrolysis method is directly proportional to the accessibility of the enzyme to the cellulose and hemicellulose components during the applied pretreatment method. Furthermore, the operating and the pretreatment condition create products that are the inhibitory effect on the downstream operations. Hydrolysis of lignocellulosic biomass commonly operated by integrated actions of cellulase enzymes or acids. These enzymes catalyze the cellulose and decrystallized with high specificity[192]. Structural performance of the substrate, including cellulose crystallinity, the accessible surface pore size area of the biomass, its degree of polymerization, and lignin content determine the exposure of the biomass to enzymatic hydrolysis 错误!未找到引用源。. Integrated action of a minimum of three enzyme groups namely, endo-glucanases, exo-glucanases, and β-glucosidases during enzymatic hydrolysis first attached on the cellulose surface through the pore followed by its degradation into principal sugar monomer and desorption of the cellulase enzyme promotes the hydrolysis of cellulose. The primary function of endoglucanases is to destruct amorphous internal regions part of the cellulose and regions of low crystallinity on cellulose fiber and generate free chain-ends. These free chain-ends are later attacked by exo-glucanases, which leads to the production of glucose and oligo-saccharide sugars (cellobiose units). Cleavage of 1,4-D glycosidic bonds in the oligo-saccharide sugars (cellobiose) is completed by β-glucosidases, which concludes the saccharification of cellulose into simple sugars [88]. Conversion of cellobiose to monomer sugars (by β-glucosidases) reduce the inhibitory effect of cellulose enzymes during enzymatic hydrolysis. (Fig. 1.3). Moreover, the integrated action of cellulases and hemicellulases has been reported for enhanced rate, and high conversion of glucan and xylan to glucose and xylulose by the improvement of accessibility of cellulases to cellulose and hemicelluloses fibrils respectively to make efficient fermentation operation for high yield cellulosic ethanol production. In general efficient cellulase hydrolysis has been obtained using an integrated research approach on cellulase enzymes using potential innate cellulase produced and adapted thermotolerant strains improved the cellulosic ethanol yield and reduced the operation cost significantly.

1.2.4. Fermentation

The production of ethanol from lignocellulose biomass is performed by one of the processing option using Simultaneous saccharification and fermentation (SSF). The principal benefits of performing the enzymatic hydrolysis together with the fermentation, instead of in

separate hydrolysis are reduced end-product inhibition of the enzymatic hydrolysis, and the reduced investment costs 错误!未找到引用源。. However, simultaneous saccharification and fermentation operation need to find favorable conditions (such as temperature and pH) for both the enzymatic hydrolysis and the fermentation have been difficult to operate at different temperature and pH conditions using fermenting organism and the enzymes. To reduce the capital investment and to simplify the fermentation process, two steps should be integrated into one pot using the simultaneous scarification and fermentation (SSF), where enzyme and fermenting microorganisms are mixed in the same bioreactor. However, there were three major obstacles for efficient integrated SSF operations, the first, in product inhibition of the cellulase enzyme during prehydrolysis caused by the oligosaccharide compound the so-called cellobiose. Cellobiose needs supplementation of additional external cellulase β-glucosidase enzyme. The second, major challenge is the usage of two types of equipment, one for hydrolysis and the other for fermentation and the third utilization of freshwater for cooling after pre-hydrolysis at high temperature. The first major challenge was solved by using innate β-glucosidase producing potential strain *Clavispora* NRRL Y- 50464 operated by a bioreactor with a helical stirring apparatus. In this study, the newly strain *Clavispora* produced a sufficient amount of cellulase to degrade the oligosaccharide cellobiose without the addition of external enzyme with low cellulase dosage at high solid loading (Chapter 2). The second and third major challenges were solved by the newly adapted thermotolerant strain *S. cerevisiae* Z100.In this practical study, the adapted strain grow at high temperature and solid loading and produced sufficient amount of cellulosic ethanol compared to the parental stain and the operation was not utilized freshwater for cooling the bioreactor after prehydrolysis (Chapter 3). The current study showed that the two potential strains improve the above limitations efficiently one by conversion of cellobiose into sugars using *Clavipora* strain and the other by adapted *S. cerevisiae* Z100 improved the one-pot SSCF operation at high temperature and solid loading without using freshwater for cooling. While it introduces CBP based operations by producing efficient cellulosic ethanol at high solid loading. In general in both practical studies consolidate bioprocessing (CBP) has been encouraged by the following great achievements, the first using innate cellulase enzyme-producing strains for the reduction of external cellulase addition, lowering cellulase dosage, The second, using adapted strain used in one pot fermentation by avoid the usage of cellulase enzyme during cell culture, eliminate contamination, avoiding the usage of fresh water for cooling, reducing cell death during temperature fluctuation and making zero wastewater generation. One-pot fermentation using lignocellulose materials by potential strain *Clavipora* and adapted thermotolerant strains *S. cerevisiae* Z100 contributing the successful application of advanced CBP technologies. The following are CBP base cellulosic ethanol production criteria's [89]-[91]. (1) Efficient enzymatic hydrolysis using innate cellulase enzyme-producing strain, (2) Thermotolerance ability for hydrolysis and fermentation at the same operations by utilizing pentose and hexose sugars, and (3) fermentation of pentose and hexose sugars into ethanol and other by-products such as glycerol. The current study, in this regard, contributes much for further large scale cellulosic ethanol production using this advanced technology.

1.3 Improved cellulosic ethanol with a low cellulase input using potential strains

β-Glucosidases (β-D-glucopyranoside glucohydrolase) [E.C.3.2.1.21] is one of the cellulase enzymes which break the β-1,4D-glycosidic bond of a carbohydrate moiety to discharge non reducing terminal glycosyl residues, glycoside, and oligosaccharides [92][93]. β-glucosidases are the essential part of the cellulase system (cellulose metabolizing enzymes) and catalyze the last and final step in cellulose hydrolysis. Cellulase enzymes hydrolyze the cellulose to yield cellobiose and other short oligosaccharides which are finally hydrolyzed to glucose by β-glucosidase. (Fig.1.3).The extra cost of cellulase cocktails is a major issue, which is necessary to deconstruct the cellulose structure and release fermentable sugars for ethanol production. For conventional corn-based ethanol technology, the expense of a fermentation cocktail is less than 3% of the total operation cost. In contrast, the cellulase cocktails currently used in the cellulosic ethanol production cost more than 30% of the total operation [94][95][94]. Reduction of the production cost, especially for the expenses of the digestive enzyme, is vital for a viable and efficient cellulosic ethanol production from lignocellulosic materials. Since β-glucosidase plays a major role in cellulose deconstruction, recently, research on β-glucosidase has drawn increased attention due to the rise of a lignocellulosic biomass-based economy [96][97][96]. For the second generation renewable cellulosic ethanol production from lignocellulosic materials, it is mandatory to add external β-glucosidase since most ethanologenic organisms are unable to degrade this the oligo-saccharide sugar (cellobiose) used in an economic simultaneous saccharification and fermentation (SSF) process. Cellulase enzymes of the conventional commercial products are the ability to digest high molecular weight cellulose but lack effective integration of cellulase action on it. Thus the β-glucosidase activity is needed to complete the enzymatic hydrolysis for cellulosic oligosaccharide sugar component-to-simple sugar conversion. Naturally occurring cellulolytic microorganisms often do not produce a significant amount of ethanol from the cellulose. On the other hand, the conventional ethanologenic agent is unable to produce sufficient β-glucosidase and an external source of β-glucosidase is vital to complete the cellulose deconstruction for microbial fermentation. The high cost of the enzyme is a major bottleneck for sustainable cellulosic ethanol production. For economic reasons, a practice using combined cellulolytic microorganisms and the ethanologenic agent was also observed. However, the extra microorganism ingests additional carbon source and lessen the final product and fermentation productivity. Recently, significant recombinant DNA technology efforts have been taken to increase cellulolytic enzyme production and support ethanologenic microbes to produce β-glucosidase [96][97]. For example, cellulolytic enzyme yield significantly enhanced for a non-fermentation fungus *Neurospora crassa* by genetic engineering [98][99]. *Saccharomyces cerevisiae* was enabled to utilize cellulose to produce cellulosic ethanol [100]. Besides, *S. cerevisiae* was also able to produce cellulase enzymes by using the genetic engineering genes encoded for cellobiohydrolases from *Aspergillus aculeatus* and *T. reesei* [101]. However, significant challenges leftover since the enzyme yield and the rate of change obtained so far are

not yet satisfactory for potential industrial applications.

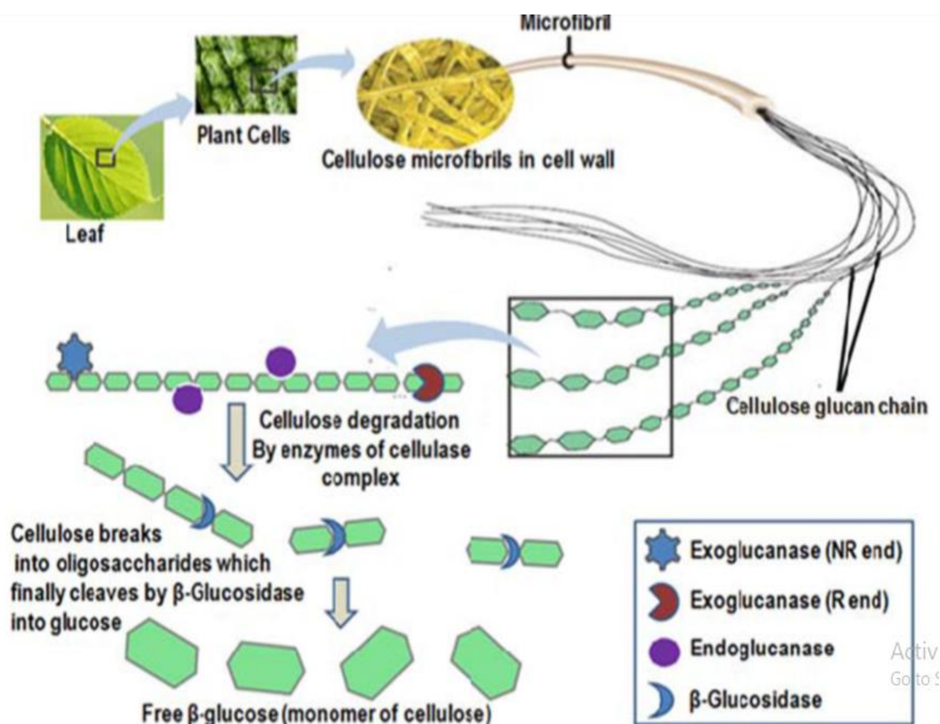


Fig. 1.3 A diagrammatic overview of cellulose metabolism by cellulase system. These smaller molecules are finally utilized by β-glucosidase as a substrate to release glucose as the final product of complete hydrolysis of cellulosic substance [193].

1.4 High temperature and solid content used for fermentation

Enzymatic hydrolysis performed at high temperature and high solids contents bargain several benefits over moderate temperature and low solid contents, the main one has improved cellulase activity and higher sugar concentrations [102] [44]. High lignocellulosic operation cost is one of the technical obstacles for its commercialization. From a cost-effective and sustainable cellulosic ethanol production point of view, the operations from pretreatment up to downstream processing of ethanol production the fermentation should be operated at high solids contents[10][11][36][37][103]. Theoretically, higher solid content and high temperature improved the sugar yield, cellulase activity and reduced the wastewater generations and contamination results higher ethanol titer, and consequently, energy consumption at the distillation stage and freshwater supplementation for cooling the bioreactor could be highly reduced. However, to operate the fermentation at high temperature and high-solids content, various limitations of lignocellulosic-based ethanol production should be addressed. These factors, including enzyme cost, xylose utilization ability, enzyme activity and its dosage, and temperature fluctuation between pre-hydrolysis and the strain to be used for fermentation around 30 ºC and pretreatment inhibitors [104]. Though, temperatures between 38 °C and 42 °C permit significant reductions of production costs [105] temperatures 50 ℃ and above would be more desirable [106]

1.5 Adaptive evolution improves the xylose utilization

The renewable biofuels by first-generation biofuel production approach using readily available feedstocks such as sugarcane juice and corn grains have been received less attraction due to its negative implication in the food versus fuel debate. On the other hand, bioethanol produced from renewable lignocellulosic feedstocks has received much attention due to their promising potential role to replace conventional fuels [74]. Currently, researchers and businesspersons are aiming at lignocellulosic biomass (LCB) which contains lignin, cellulose, and hemicelluloses, where, cellulose and hemicellulose undergo hydrolysis yielding fermentable sugars like glucose and xylose [107][108]-[113]. Besides, the biochemical productions using pentose sugars like xylose which is the second most abundant sugar, making 5–35 % share of total dry LCB encouraging the production at a large scale level [114][116][114]. Moreover, both pentose and hexose sugars must be utilized for sustainable and affordable economical production of lignocellulosic bioethanol [117][118].[117] The yeast *Saccharomyces cerevisiae* has traditionally been used and remains the organism of choice for large-scale industrial ethanol production from hexose. However, the adapted thermotolerant strain *S.cerevisae* XH7 does not significantly metabolise xylose in our practical study, which was the second major constituent of the of lignocellulosic feedstock and has become an attractive raw material for ethanol production due to its abundance and low cost compared to starch and sucrose-based materials. More than a decade of research has devoted to the development of yeast strains for efficient xylose fermentation, with major efforts focused on functional expression of bacterial and fungal xylose-utilizing genes and handling the pentose phosphate pathway to enhance xylose utilization and fermentation in *S. cerevisiae* [4] The traditional yeast *Saccharomyces cerevisiae* is efficient to ferment glucose but unable to ferment xylose for ethanol production [119] .Xylose fermentation and subsequent ethanol production have been studied in several xylose-fermenting yeasts, including *Scheffersomyces* (Pichia) *stipitis, Candida shehatae*, *Pachysolen tannophilus* and *Kluyveromyces marxianus* [116]. However, their ethanol productivities are very poor as compared to *S. cerevisiae* when glucose-based substrates are used[120]. There are some other limitations, including ethanol and sugar tolerance, undesired by-product formation, limited substrate specificity, redox imbalance and low ethanol productivity[121]. The majority of the studies have conducted using metabolic engineering through a rational selection of genes to be manipulated for the development of novel pentose-fermenting strains with varying levels of success[122]. However, the mechanisms by which pentose fermenting yeasts accomplish to increase the rate of ethanol production are still not fully explored. The simultaneous co-fermentation of hexose and pentose sugars also constitutes a major challenge in strain development[123]. Efforts have also been made to establish a xylose-utilizing pathway in *S. cerevisiae* by insertion of the genes encoding xylose reductase, and xylitol dehydrogenase from *S. stipitis* and xylose isomerase(XI) from bacteria and fungus resulted in poor ethanol production from xylose together with undesired metabolites.In this practical study the parental strain S. cerevisiae XHZ is derived from the wild-type diploid *S. cerevisiae* strain SF through rationally designed genetic modifications is able to degrade xylose using the enzyme xylose

reductase(XR) produces xylitol and xylitol converted into xylulose by xylitol dehydrogenase(XDH) produces xylulose again converted into Xylulose-5-phosphate, using the enzyme Xylulose kinase (XK) in the PPP pathway then converted into other intermediate molecules such as Fructose-6-phosphate and Glyceraldehyde-3-phosphate and following steps produce ethanol. Major attention has been paid to *S. cerevisiae* and other mesophilic yeast strains *including S. stipitis* and *C. intermedia* for development of a genetically modified model for ethanol production from lignocellulosic biomass[116]However, mesophyll yeast strains have a drawback in saccharification and fermentation at low temperatures, in terms of energy requirements for mixing and product recovery, which increases the cost of operations [126][127]. On the other hand, hemophilic and thermotolerant yeast strains have many advantages over mesophylls, i.e. higher saccharification and fermentation rate, broad substrate utilization range together with less energy requirement for mixing and product recovery, lower cost of pumping and steering, no cooling required and minimized contamination risk [109][128]. *K. marxianus* carrying all abovementioned advantages and will be the better alternative to *S. cerevisiae* and *S. stipitis* for the ethanol production process shortly [128]-[131]. But the current study showed that newly adapted thermotolerant strain *S. cerevisiae* Z100 in this study has been much better than *K. marxianus*. Evolutionary adaptation is an important strategy to enhance strains with desired production traits. Various researchers have employed this strategy to generate improved xylose-fermenting strains [134]-[137]. The potentially adaptive evolution has been elucidated by several researchers in improving the growth and fermentation process of microorganism when xylose is used as the sole source of carbon [133][136]. The adaptation evolution fermenting microorganisms used to inhibitory lignocellulosic hydrolyzates is another possible tool for solving the inhibitor problems. Adaptive evolution has been employed to the recombinant microbial strains to increase their fermentation capability. However, this strategy does not affect any genetic changes [138]. One method which allows the selection of simultaneous genetic modificationsin-network pathways to improve its performance is evolutionary engineering, involving reprogramming the cell's natural metabolic pathway at different levels. This method was based on adaptive evolution, a natural operation in which organisms undergo mutations that confer tolerance to environmental changes [139]. However, Adaptive natural adaptive evolution is slow, but there are means to accelerate this process, mainly for microorganisms because of their fast growth. The microorganism is placed under environmental selection, and after some time the survived population will exhibit the desired phenotype due to "natural" mutations which permitted adaptation. These mutations enhance the expression of a certain set of genes that increase xylose utilization [140]. Evolutionary adaptation has many advantages; (1) increase temperature tolerance, (2) improve fermentability, and xylose utilization, and (3) reduce cellulase enzyme dosage during seed culture adaptation, *S. cerevisiae* Z100 used in this study were experienced a long term evolutionary adaptation by using wheat straw solids. The present study was explored an improved xylose utilizing strain for lignocellulosic bioethanol production having improved xylose uptake rate in *S. cerevisiae* Z100 through evolutionary adaptation strategy. The xylose utilization potentials at a high temperature of the adapted strain have also been reported and compared with the parental strain.

1.6 Objectives of the thesis

The main objectives of this practical study were to study on cellulosic ethanol fermentation improvement by cellulase producing and thermotolerant yeast strains the detailed objectives of the thesis are as follows:

- To determine the effect of the β-glucosidase enzyme on cellulosic ethanol production using newly strain *Clavipora* NRRL Y-50464 and *S. cerevisiae* DQ1 at a high solid loading of SSF and SHF condition (Chapter 2)
- To improved cellulosic ethanol production from corn stover with a low cellulase input using a β-glucosidase-producing yeast following a dry biorefining process (Chapter 2).
- To evaluate the cellobiose utilization potential of newly *Clavipora* NRRL Y-50464 strain as compared to Commonly used *S. cerevisiae* DQ1 (Chapter 2)
- To evaluate newly developed adapted thermotolerant and xylose utilized strain of *Saccharomyces cerevisiae* Z100 thermotolerance potential (Chapter 3).
- To determine the xylose utilization potential of the adapted thermotolerant strain, by adaptive evolutionary laboratory method using freshly solid wheat straw feedstock without cellulase. (Chapter 3)
- To evaluate the ethanol production and xylose utilization potentials of adapted thermotolerant strain *S. cerevisiae Z100* potentials compared to the parental strain *S. cerevisiae* XH7 (Chapter 3)

We believe these Practical study result would contribute a lot for the development of cellulosic ethanol production in large scale using lignocellulosic biomass in the future.

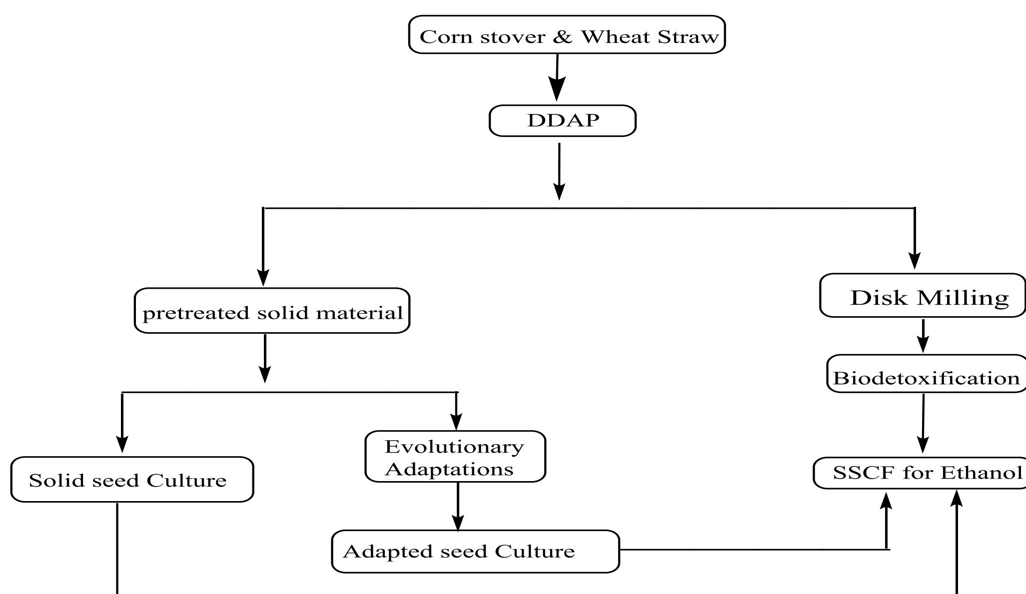


Fig. 1.5 The whole experimental graphics of the research work in this thesis

Chapter 2

Improved cellulosic ethanol production from corn stover with a low dosage cellulase input using a β-glucosidase-producing yeast strain following a dry biorefining process

2.1 Introduction

Renewable biofuels, including cellulosic ethanol, are attractive alternatives as transportation fuels to reduce the use of fossil fuels that aid mitigation of greenhouse gas emissions. Significant development has been made in the past decades toward commercialization of cellulosic ethanol production [141].[141]However; challenges remain for sustainable and economical operations in full commercial scales [142]. The extra cost of cellulase cocktails is a significant issue, which is necessary to deconstruct the cellulose structure and release fermentable sugars for ethanol production. For conventional corn-based ethanol technology, the expense of a fermentation cocktail is less than 3% of the total operation cost. In contrast, the cellulase cocktails currently used in the cellulosic ethanol production cost more than 30% of the overall operation [94][95][94]. Reduction of the production cost, especially for the expenses of the digestive enzyme, is vital for a sustainable and competent cellulosic ethanol production from lignocellulosic materials. Since most cellulase complexes produced from bacteria and fungi have insufficient β-glucosidase activity, a supplement of external β-glucosidase has been required during enzymatic hydrolysis for cellobiose-to-glucose degradation[143][144], significant efforts had been taken to enable ethanologenic yeast to secrete or tether β-glucosidase on the cell surface through genetic engineering approaches [82][145][82]. However, adequate β-glucosidase activity and the rapid enzymatic hydrolysis process require a massive amount of mutant yeast cells, which is impractical for commonly applied simultaneous saccharification and fermentation (SSF) processes [82]. A recently reported strain *Clavispora* NRRL Y-50464 is a natural β-glucosidase-producing yeast which produces at least three forms of β-glucosidases, BGL1, BGL2 and BGL3 [146]. It has been demonstrated to produce cellulosic ethanol from lignocellulosic materials without the addition of extra β-glucosidase via SSF [147][148][147]. However, the previously reported process was less efficient in application with a relatively higher cellulase dose at 34 FPU/g corncob residues or 15.3 FPU/g corn stover biomass using Celluclast 1.5 L, a commercial cellulase without β-glucosidase activity. The lower efficiency was partially attributed to the conventional bioreactor commonly used for liquid fermentation, which is inefficient for cellulosic ethanol production from a slurry of cellulosic solids. The need for process engineering, including a different design of a suitable bioreactor for cellulosic ethanol production using SSF is realized [148]. After preliminary evaluations, we found that it was promising to obtain a more efficient ethanol fermentation using this β-glucosidase producing strain with a low cellulase dose. Here, we applied a bioreactor with a helical stirring apparatus that provides sufficient mixing power and mass

conveying capability during enzymatic hydrolysis for higher levels of cellulosic solids loading [149]. This process is currently the most suitable and more efficient for cellulosic ethanol production using SSF. In this study, we characterised ethanol production from corn stover using the β-glucosidase-producing strain *Clavispora* NRRL Y-50464 with a lower cellulase input at 5 mg protein/g glucan. The evaluation was compared using an ethanologenic yeast *Saccharomyces cerevisiae* as control under both separated hydrolysis and fermentation (SHF) and SSF conditions.

2.2 Materials and Methods

2.2.1 Raw materials

Corn stover was obtained from Bayan Nur League, Inner Mongolia Autonomous Region, China. The collected corn stover was milled using a beater pulverizer (SF-300, Ketai Milling Equipment, Shanghai, China) to pass through a mesh with a diameter of 10 mm. The milled corn stover was stored under dry conditions in plastic bags until use. A commercial cellulase enzyme powder Youtell #7, and a liquid β-glucosidase enzyme Youtell #184 were provided by Hunan Youtell Biochemical Co. (Yueyang, Hunan, China). The enzyme activity was evaluated using an NREL protocol LAP-006 and a method, as previously described [150][151][150]. Youtell #7 had a cellulase activity of 63.0 FPU/g and a β-glucosidase activity of 99.9 CBU/g. Youtell #184 had a β-glucosidase activity of 5678 CBU/mL. The protein content was determined by the Bradford method with bovine serum albumin (BSA) as a protein standard for Youtell #7 and #184 as 46.7 mg/g and 40.0 mg/g, respectively [152].

2.2.2 Enzymes and culture media

A β-glucosidase-producing yeast *Clavispora* NRRL Y-50464 was obtained from ARS Patent Culture Collection, Peoria, IL USA. Cell cultures were maintained and precultured on a medium containing 50 g glucose, 3.0 g yeast extract, and 5.0 g peptone per liter. The fermentation or SSF operation was carried out on a medium with the following nutrient supplementations: 3.0 g/L yeast extract, 5.0 g/L peptone, 2.0 g/L KH_2PO_4, 1.0 g/L $(NH4)_2SO4$, 0.5 g/L $MgSO_4•7H_2O$, 0.1 g/L NaCl, and 1.0 mL/L of a trace element solution. The trace element solution consisted of 0.25 g $CuSO4•5H_2O$, 0.169 g $MnSO_4•H_2O$, 0.287 $ZnSO_4•7H_2O$, and 0.238 $CoCl_2•6H_2O$ per litre [147]. A thermotolerant strain *Saccharomyces cerevisiae* DQ1, also known as CGMCC2528 from China General Microorganism Collection Center, was used as a control [153] *S. cerevisiae* DQ1 is unable to metabolize cellobiose into ethanol. For this stain, a supplement of nutrients, including 2.0 g/L KH_2PO_4, 1.0 g/L $(NH_4)_2SO_4$, 1.0 g/L $MgSO_4•7H_2O$, and 1.0 g/L yeast extract, was applied into the fermentation system for ethanol production [153].

2.2.3 Dry acid pretreatment and biodetoxification

Corn stover was pretreated using a dry acid pretreatment method, as described previously [154][155][154]. Briefly, the solid corn stover and the liquid dilute sulfuric acid (7.2%, w/w) were concurrently fed into the pretreatment reactor (pCF20-1.6, Keli Chemical Equipment, Yantai,

China) at a ratio of 2:1. The mix was incubated at 175 ℃ for 5 min under mild helical agitation. The pretreated corn stover contained 38.0% of glucan and 4.4% of xylan as analyzed following a two-step dilute sulfuric acid hydrolysis procedure [156].The solid content of the pretreated corn stover was about 50%. Then, the pretreated corn stover was neutralized to pH 5.0 with 20% (w/w) calcium hydroxide slurry and disk milled to remove the long cellulose fibres. It was further incubated in a 15-L bioreactor at 28 ℃ and aeration for 48 h to remove the inhibitory compounds generated during the dry acid pretreatment procedures as previously described [157][158][157].There was no wastewater generated during the process of pretreatment and biodetoxification treatment. But a substantial amount of monomer xylose was consumed by *A. resinae* ZN1 after the inhibitors were degraded [157].

2.2.4 Enzyme hydrolysis

The pretreated corn stover was enzymatically hydrolyzed at 25%, 30%, and 35% solids loading (w/w) separately in a helical stirring bioreactor at 50 ℃ for 48 h with Youtell #7 at a dose of 5 mg protein/g glucan (equal to 6.7 FPU/g glucan). The pH was maintained automatically at 4.8 with 20% (w/w) sodium hydroxide. No antibiotics were incorporated in any phase of this study. The slurry of the hydrolyzed corn stover was centrifuged at 10,000 rpm for 10 min to obtain a clear corn stover hydrolysate to be used for the following fermentation experiments. Such obtained corn stover hydrolysate (CSH) at 25% solids loading (25% CSH) contained 57.8 g/L glucose, 15.6 g/L xylose, 13.4 g/L cellobiose, and 1.2 g/L acetic acid. The 30% CSH contained 66.2 g/L glucose, 15.4 g/L xylose, 14.1 g/L cellobiose, and 2.5 g/L acetic acid. The 35% CSH contained 79.6 g/L glucose, 13.2 g/L xylose, 16.5 g/L cellobiose, and 4.0 g/L acetic acid.

2.2.5 Simultaneous saccharification and fermentation

The SSF operation was carried out in a 5-L bioreactor with a helical stirring apparatus, as described previously [149]]. The pretreated and bio-detoxified corn stover and the bioreactor used during SSF were autoclaved at 121 ℃ for 20 min. The corn stover was first pre-hydrolyzed to a liquid state at 25% (w/w) solids loading with 5 mg protein/g glucan of Youtell #7 at 50 ℃ with agitation at 150 rpm for 12 h at pH 4.8. Then, an inoculation was made at a 10% ratio (v/v) using a seeding yeast cultivated overnight. The SSF was maintained at 37 ℃ and the pH adjusted at 5.5 by automatic regulation with 5 M NaOH. For the SSF without β-glucosidase addition, no extra β-glucosidase was supplemented to the system. However, for the SSF with β-glucosidase addition, extra β-glucosidase Youtell #184 was added to the system at the beginning of pre-hydrolysis with a dose of 91.1 CBU/g glucan. Samples were taken periodically, and supernatants were obtained by centrifugation at 13,000 rpm for 5 min. Fermentation profiles were analyzed using HPLC, as previously described [159].

2.2.6 Analysis

Glucose, xylose, cellobiose, acetic acid, and ethanol in a sample were analyzed on HPLC (LC-20AD, Shimadzu, Kyoto, Japan) equipped with a Bio-rad Aminex HPX-87H column (Bio-

rad, Hercules, CA, USA) and RID-10A detector (Shimadzu, Kyoto, Japan). A solution of 5 mM H_2SO_4 was used as eluent with a flow rate of 0.6 mL/min. The cellulose conversion efficiency (as ethanol yield) was calculated using the method described by Zhang and Bao specifically for the high solid SSF process [160]:

$$\text{Cellulose conversion efficiency} = \frac{[C1 \times W]}{976.9 - 0.804 \times [C1]} \times \frac{1}{0.511 \times f \times [biomass] \times m \times 1.111} \times 100$$

where [C1] stands for the ethanol concentration in the culture broth (g/L), W is the total water input of SSF (g), f the cellulose fraction of corn stover feedstock, [Biomass] the dry corn stover concentration at the beginning of SSF (g/g), m the total weight of SSF (g), 0.511 the conversion factor for glucose to ethanol based on stoichiometric biochemistry of yeast, and 1.111 the conversion factor for cellulose equivalent to glucose

2.3 Results and Discussions

2.3.1 Cellobiose assimilation of *Clavispora* NRRL Y- 50464

The cellobiose assimilation capacity of *Clavispora* NRRL Y-50464 was evaluated in comparison with *S. cerevisiae strain* DQ1 using either glucose or cellobiose as a sole carbon source. On a medium using glucose as the sole carbon source, both strains were able to grow a cell mass, consume the sugar, and produce ethanol. However, *Clavispora* NRRL Y-50464 showed a significantly faster rate of cell growth and glucose consumption and produced almost the same amount of ethanol compared with *S. cerevisiae* DQ1 (Fig. 2.1a). Under cellobiose as the sole carbon source conditions, *S. cerevisiae* showed a minimum cell growth likely from the trace amount of glucose that existed in the yeast extract (Fig. 2.1b). It did not produce any ethanol, and the cellobiose remained intact in the medium unutilized. In contrast, *Clavispora* NRRL Y-50464 displayed normal cell growth and depleted cellobiose in no more than 12 h for ethanol conversion (Fig. 2.1b). According to our, previous studies, a large amount of β-glucosidase activity (specific activity of 1.20 U/mg/mL) was observed in crude cell protein extracts by in vitro assay when the strain Y-50464 was grown on cellobiose [147]. Thus, *Clavispora* NRRL Y-50464 was capable of transforming cellobiose into ethanol directly, which has potential in reducing the use of additional β-glucosidase, a significant component of cellulase, for lower-cost cellulosic ethanol production.

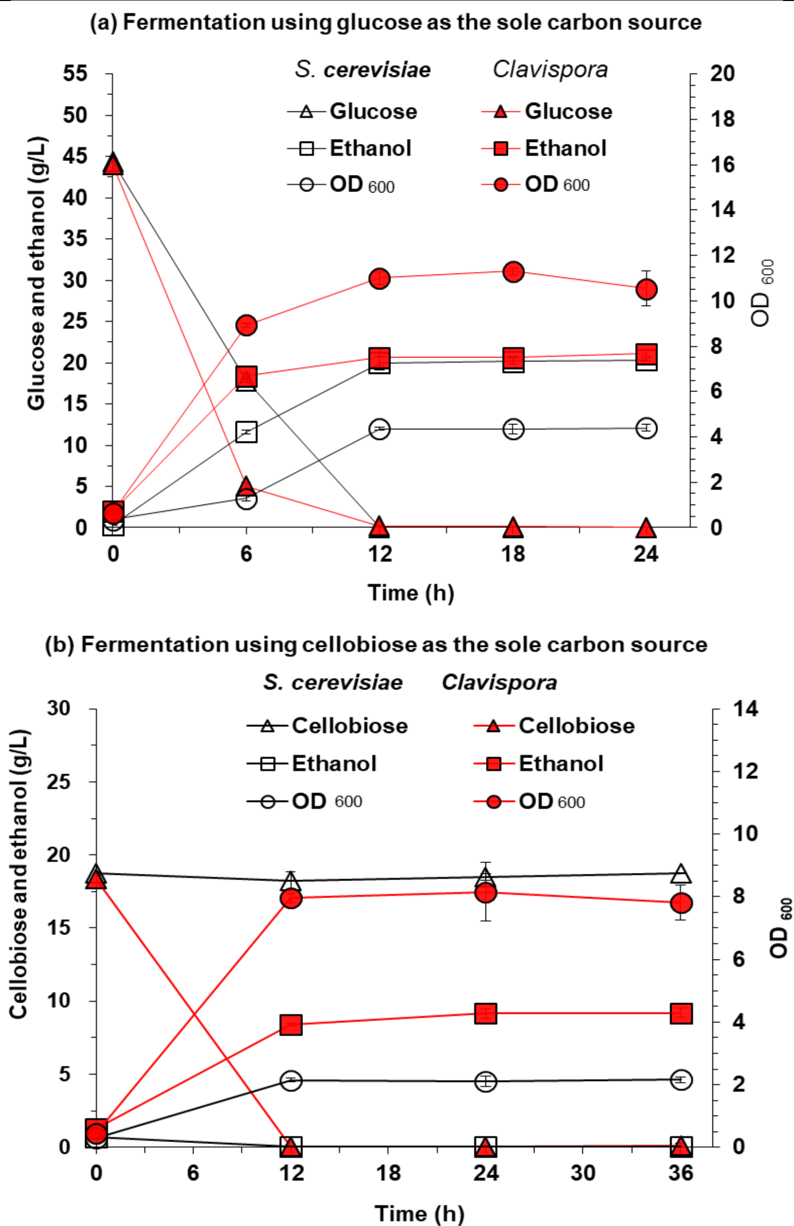


Fig. 2.1 Comparison of ethanol fermentation profiles between *S. cerevisiae* DQ1 and *Clavispora* NRRL Y-50464 in response to different sugar conditions. (a) Fermentation using glucose as the sole carbon source; and (b) fermentation using cellobiose as the sole carbon source. Fermentation conditions: 37 ℃ for *Clavispora* NRRL Y-50464 and 30 ℃ for *S. cerevisiae* DQ1 in a shaking incubator with the agitation speed of 150 rpm.

2.3.2 Ethanol production from corn stover hydrolysate

The corn stover hydrolysate (CSH) was prepared at 25%, 30%, and 35% solid loading (w/w) separately. Following a previously reported process economic analysis [94] a cellulase dose of 5 mg protein/g glucan was applied in this study. Since Youtell #7 was observed to possess a low β-glucosidase activity (99 CBU/g), the product inhibition of glucose on cellulase was more serious at a higher solids loading of corn stover. As a result, cellobiose accumulation in the CSH increased with the increase in the solids loading. The final cellobiose concentration of the CSH samples was 13.4, 14.1, and 16.5 g/L for solids loading of 25%, 30%, and 35%, respectively. The cell growth of *Clavispora* NRRL Y-50464 was significantly higher than that of *S. cerevisiae* DQ1 as measured by OD_{600} for all three solids loading levels (Fig. 2.2a). Both strains showed a decreased trend of cell density with increased levels of solids loading in CSH. Acetic acid was observed at 1.2, 2.5, and 4.0 g/L for the solids loading in CSH of 25%, 30%, and 35%, respectively. The decreased cell growth under higher levels of solids loading was possibly due to the higher acetic acid and phenolic acid concentrations accumulated in the CSH. *Clavispora* NRRL Y-50464 produced significantly higher levels of ethanol for all solids loading CSH compared with those fermented by *S. cerevisiae* DQ1 (Fig. 2.2b). The ethanol production increased with the increase of the solids loading levels at 26 g/L for 25% CSH and with the highest of 38 g/L for 35% CSH. The increased portion of ethanol production was attributed to the cellobiose in the CSH since strain Y-50464 is able to convert cellobiose into ethanol directly. *Clavispora* NRRL Y-50464 showed about 80% of cellobiose consumption and, as anticipated, *S. cerevisiae* strain DQ1 did not utilize any cellobiose at all (Fig. 2.2c). For SHF at 25% (w/w) solids loading, the cellulose conversion efficiency was about 51% during the enzymatic hydrolysis step. The ethanol metabolic yield was 80% and 65% in the fermentation step for strain Y-50464 and strain DQ1, respectively. Thus, the net ethanol production was less than 41% for strain Y-50464 and 33% for strain DQ1 from cellulose in the corn stover. The lower efficiency of enzymatic hydrolysis was partially attributed to the lower input of cellulase at 5 mg protein/g glucan and the product inhibition in the presence of high glucose concentration. In addition, a residue amount of xylitol as a by-product was observed from cellobiose conversion by strain Y-50454 [148]. Thus, the cellobiose-to-ethanol pathway in Y-50464 also needs to be optimized in the future for more efficient cellobiose conversion.

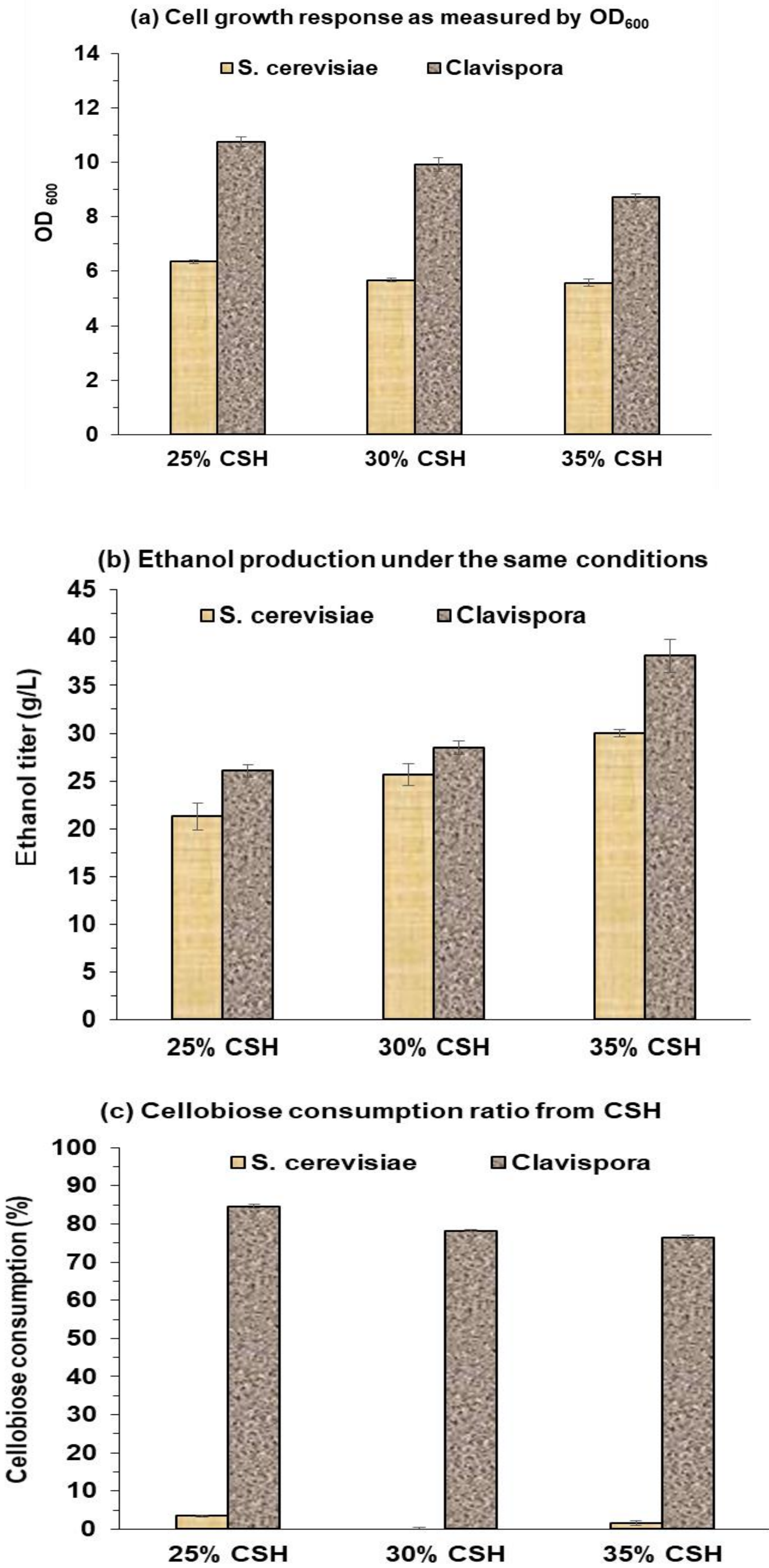

(a) Cell growth response as measured by OD_{600}
S. cerevisiae
Clavispora
OD_{600}
14
12
10
8
6
4
2
0
25% CSH
30% CSH
35% CSH
(b) Ethanol production under the same conditions
S. cerevisiae
Clavispora
Ethanol titer (g/L)
45
40
35
30
25
20
15
10
5
0
25% CSH
30% CSH
35% CSH
(c) Cellobiose consumption ratio from CSH
S. cerevisiae
Clavispora
Cellobiose consumption (%)
100
90
80
70
60
50
40
30
20
10
0
25% CSH
30% CSH
35% CSH

Fig. 2.2 Comparison of ethanol fermentation performance between *S. cerevisiae* DQ1 and *Clavispora* NRRL Y-50464 from corn stover hydrolysate prepared at different solids loading levels at 25%, 30% and 35% (w/w). (a) Cell growth response as measured by OD600; (b) ethanol production under the same conditions; and (c) Cellobiose consumption ratio from the corn stover hydrolysate. Fermentation conditions: 37 ℃, 150 rpm in a shaking incubator using 25%, 30%, and 35% CSH, respectively

2.3.3 Ethanol production from SSF

The SSF procedure is commonly applied for cellulosic ethanol production. It has an advantage in alleviating the glucose product inhibition on cellulase by converting the glucose into ethanol as it was released to reduce sugar accumulation. As a result, a higher ethanol yield often can be obtained relative to the operation using a separated hydrolysis and fermentation process [161]. In this study, we conducted comparative SSF for both strains applying 25% solids loading (w/w) of corn stover treated with 5 mg protein/g glucan using Youtell #7. For *S. cerevisiae* DQ1, ethanol production was lower over time without the addition of β-glucosidase compared with that of added β-glucosidase treatment. In the absence of extra β-glucosidase, an ethanol titer of 30.0 g/L was obtained at 96 h after incubation.

Table 2.1 Comparison of ethanol production from corn stover at 25% solids loading (w/w) between *S. cerevisiae* DQ1 and *Clavispora* NRRL Y-50464 using SSF

Strains	β-glycosidase	Ethanol (g/L)	Cellulose conversion yield (%)
S. cerevisae	Not added[a]	30.3±0.6	44.1±0.1
S. cerevisae	Added[b]	36.9± 0.1	53.7±0.1
Clavispora	Not Added[a]	38.1±1.9	55.5±0.2
Clavispora	Added[b]	37.7± 0.2	54.9±0.1

[a] Cellulase Youtell #7 was added at 5 mg protein/g glucan to allow positive fermentation, but extra β-glucosidase Youtell #184 was not added.

When β-glucosidase was added in the system, the ethanol titer was increased to 36.9 g/L for *S. cerevisiae* DQ1 (Fig. 2.3a; Table 2.1). This indicated an insufficient β-glucosidase activity of Youtell #7 applied, which resulted in the inefficient synergetic effect among the cellulase components (endo-, exo-glucanase, and β-glucosidase) and the poor cellulose conversion ratio. Unlike often observed cellobiose accumulation in CSH, no significant cellobiose accumulation occurred during SSF stage, even for the treatment without extra β-glucosidase supplementation. It reflected the advantage of SSF with a better alleviation of the product inhibition on cellulase. On the other hand, the addition of extra β-glucosidase did not affect the performance of *Clavispora* NRRL Y-50464 at all. There was no significant difference in cellobiose consumption and ethanol conversion between SSF treatments with and without β-glucosidase

supplementation (Fig. 2.3b). The fermentation treatment without the addition of extra β-glucosidase produced an ethanol titer of 38.1 g/L with a conversion efficiency of 55.5% (Table 2.1). The treatment with added β-glucosidase generated a similar amount of ethanol with a titer of 37.7 g/L and conversion efficiency of 54.9%. Performance of both treatments by strain Y-50464 surpassed the fermentation by *S. cerevisiae* under the same conditions as measured by the above parameters. These results suggested that the native β-glucosidase activity from strain Y-50464 was not only able to compensate the deficit of You tell #7, but was also sufficient, by its native enzyme production, to complete the hydrolysis of cellobiose for ethanol conversion.

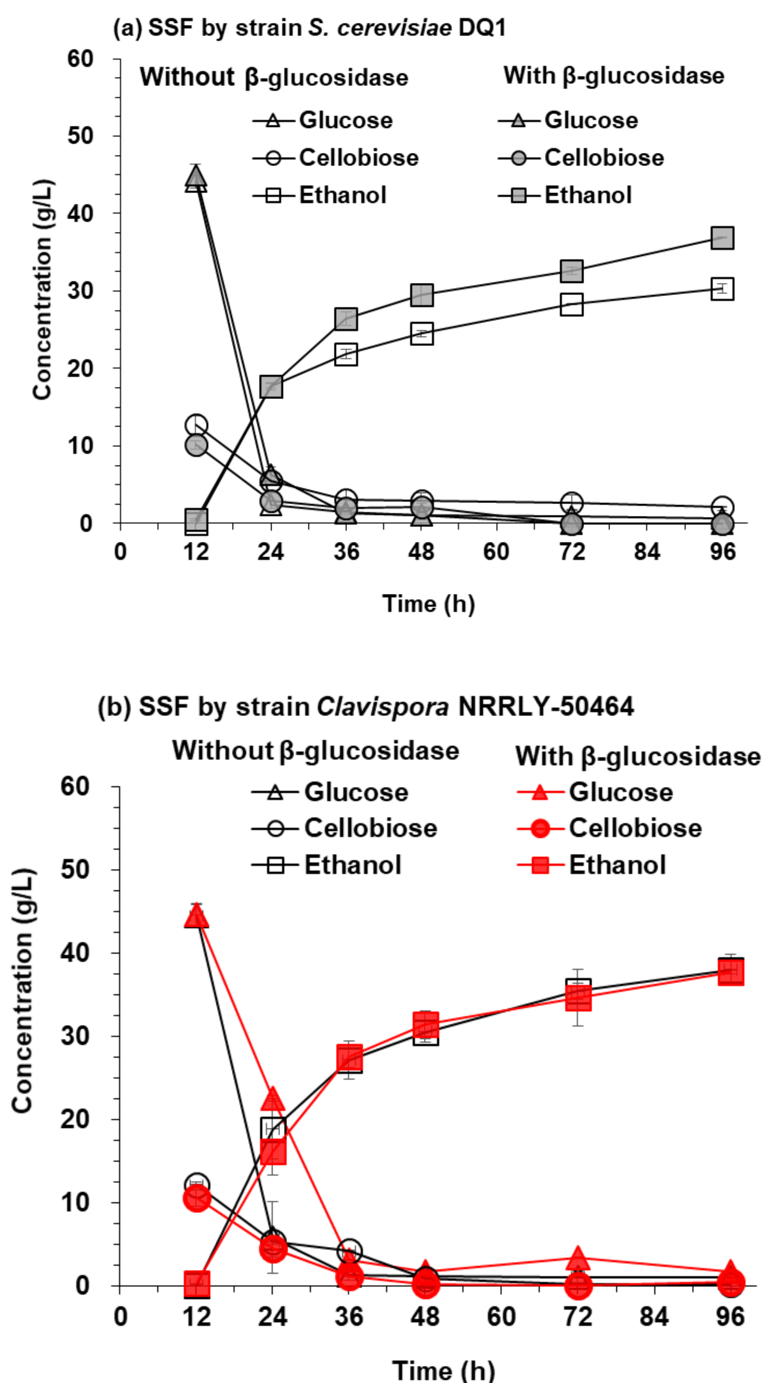


Fig. 2.3 Sugar conversion performance of simultaneous saccharification and fermentation from corn stover at 25% (w/w) solids loading. (a) Strain *S. cerevisiae* DQ1 performance with or without the addition of β-glucosidase in the medium; and (b) Strain *Clavispora* NRRL Y-50564 performance with or without the addition of β-glucosidase in the medium. Conditions: prehydrolysis stage was maintained at 50 °C, p^H 4.8 for 12 h, while the SSF stage was maintained at 37 °C, pH 5.5 for 84 h. Yeast seeds were inoculated at a ratio of 10% (v/v).

2.3.4 Ethanol production potential using strain Y﹣50464

Using a commercial cellulase Celluclast, 1.5 L which lacks β-glucosidase activity, at a dose of 34 FPU/g, ethanol production of 23 g/L was obtained previously from corncob residue without the addition of β-glucosidase by SSF using *Clavispora* NRRL Y-50464 [147]. By applying similar conditions, only 17.2 g/L ethanol was obtained from conventional corn stover from NREL-DOE at 25% solids loading with an efficiency of 32.4% [148]. In this study, using a very low dosage of 5 mg protein/g glucan from Youtell #7 (equal to 6.7 FPU/g glucan), we obtained a significantly higher ethanol production of 38.1 g/L from 25% solids loading. It also significantly improved the conversion efficiency of up to 55.5%. Such a significant improvement is largely attributed to the bioreactor applied in this study, which has a special helical stirring apparatus that provides a strong mixing power and efficient mass transferring efficiency against the slurry of corn stover during the SSF process. Conversion of corn stover to ethanol was found to be more efficient at 15% solids loading using conventional bioreactors [148][148]. The efficiency decreased significantly with the increase in solids loading. For example, at 25% solids loading, the conversion efficiency only reached 32.4%. In this study, the conversion efficiency was 55.5% for 25% solids loading, which demonstrated the significant advantage of using the bioreactor with a unique helical stirring apparatus. Although the available sugar base from different corn stover sources varies and is difficult to compare, the large margin of the difference demonstrated by this study suggested that the helical stirring bioreactors used in this study were desirable for cellulosic ethanol production by SSF. In addition to using adapted natural isolates, genetic engineering efforts have been made to enable *S. cerevisiae* producing β-glucosidase. Ethanol production was evaluated from varied sources of cellulose, including pure commercial cellulose and raw corn stover with different cellulase inputs (Table 2.2). In general, recombinant *S. cerevisiae* strains expressing cellobiose transporter or β-glucosidase did not produce satisfactory levels of cellulosic ethanol even with higher levels of cellulase input in the fermentation. It needs to be pointed out that we applied only 5 mg protein/g glucan of cellulase in this study to achieve a high ethanol production close to the minimum industry standard of 40 g/L from genuine lignocellulosic materials such as corn stover using *Clavispora* NRRL Y-50464 without the addition of extra β-glucosidase. Three forms of β-glucosidases, BGL1, BGL2, and BGL3, were characterized by *Clavispora* NRRL Y-50464 [146] [146]. Since additional enzyme activity was observed, other new forms of the enzyme are expected to be discovered in the future. These enzymes demonstrated hydrolysis activities toward at least 14 oligosaccharide substrates, including cellotetraose, cellopentaose, laminaritetraose, laminaripentaose, lactose, lichenan and other complex oligos with glycosidic

bonds [146] Such a comprehensive hydrolysis capability clearly contributed to a broad range of the β-glucosidase activity, which is a significant component of the cellulase complex for complete deconstruction of cellulosic materials for SSF. The minimum industry standard of ethanol production is 40 g/L. *Clavispora* NRRL Y-50464 produced 38.1 g/L without the addition of extra β-glucosidase via SSF in this study, which came soon than expected.

Further improvement of the yeast performance is needed. For example, lignin was observed to inhibit cellulose-to-ethanol conversion and a dignified cellulose corn stover significantly improved ethanol production and conversion efficiency up to 65% [148] Stain Y-50464 was able to produce 40.44 g/L of the ethanol from pure cellulose using low-efficient SSF with bottles. This level of the titer is likely to be reached easily by application of the more efficient helical stirring bioreactors. In addition to converting cellobiose into ethanol, a small amount of xylitol as a by-product was also produced by strain Y-50464 at the end of SSF [147][148]. Future improvement of the cellobiose-to-ethanol conversion pathway for Y-50464 is needed to utilize accessible sugars for higher ethanol productivity efficiently. Also, strain Y-50464 is suitable for ethanol production from cellulosic solids by SSF and limited in xylose-to-ethanol conversion. Thus, it may be more useful for specific niche applications, but not fit for conventional ethanol production from lignocellulosic hydrolysates which contain a large portion of xylose. With the continued improvement of this β-glucosidase-producing yeast, it is expected to have applications in a more extended scale for cellulosic ethanol production.

Table 2.2. A summary of cellulosic ethanol production using β-glucosidase-producing strains without the addition of extra β-glucosidase

Strains	**substrate conditions**	**Cellulase dosage**	**Eth.titer (g/L)**	**Ref.**
S. cerevisae NAN 227	Corncobs/bottle/96	20 IU/g solids[a]	20	[164]
S. cerevisiae D-56	Crystalline cellulose/bottle/96 h	25 FPU/g cellulose[b]	26.37	[82]
S.cerevisae INVsc1	Japanase cedar/bottle/72 h	15 mg protein/g solids[c]	~18	[165]
S.cerevisiae SyBE001603	Avicel/bottle/144 h	10 FPU/g glucan[d]	~16	[166]
Clavispora	Corncob residue/fleaker /48 h	0.2 mL/g solids[b]	22.7	[163]
Clavispora	Rice straw/bottle/36 h	9 FPU/g solids[b]	25	[167]
Clavispora	Avicel/bottle/72 h	15.3 FPU/g cellulose[b]	40.44	[148]

Clavispora	SigmaCell/bottle/72 h	15.3 FPU/g cellulose[b]	39.64	[148]
Clavispora	Conventional NREL corn stover/bottle/72 h	15.3 FPU/g cellulose[b]	17.2	[148]
Clavispora	Dilignified NREL corn stover/bottle/72 h	15.3 FPU/g cellulose[b]	28.2	[148]
Clavispora	Conventional NREL corn stover/bioreactor/48 h	15.3 FPU/g cellulose[b]	32	[148]
Clavispora	Rice straw/bottle/36 h	12 FPU Cellic CTec	36.7	[168]
Clavispora	Corn stover/ helical stirring bioreactor/96 h	5 mg protein/g glucan (6.7 FPU/g glucan)	38.1	**In this study**

2.4 Conclusions

The β-glucosidase-producing yeast strain *Clavispora* NRRL Y-50464 exhibited a superior ethanol fermentation performance over the control strain *S. cerevisiae* DQ1 in both SHF and SSF processes. The ethanol titer of 38.1 g/L and conversion efficiency of 55.5% were obtained from 25% solids loading (w/w) with a low cellulase dose of 5 mg protein/g glucan without the addition of extra β-glucosidase. The strong native β-glucosidase activity generated from *Clavispora* NRRL Y-50464 provided sufficient complementary hydrolysis capability to reduce the enzyme cost for cellulosic ethanol production by SSF. This work also demonstrated that a bioreactor with a helical stirring apparatus is more suitable than the conventional bioreactor originally designed for liquid fermentation for improved cellulosic ethanol production by SSF. *Clavispora* NRRL Y-50464 demonstrated potential as a candidate, with future improvement, for lower-cost cellulosic ethanol production from lignocellulosic materials.

Chapter 3

Ethanol fermentation from wheat straw at high temperature using thermotolerant and xylose utilized adapted strain *S. cerevisiae* Z100

3.1 Introduction

Temperature between 25 ºC and 35 ºC to maximize ethanol production and prevent irreversible heat-inactivation of the yeast cells. On the other hand, the enzyme costs, product (sugar) inhibition and loss of productivity due to inactivation of yeast cells have been the main obstacles for cellulosic ethanol production [169][170][169]. Furthermore, most of the large-scale production of fuel ethanol typically accomplished in large-scale yeast fermentations operated at high temperatures, high glucose concentrations, and elevated ethanol titers, all conditions that cause stress for the yeast cells [171][39][105]. Besides, match of the SSF operated at optimal temperatures between the ethanologenic microorganisms used (generally below 37 ℃) and the cellulase enzymes (50 ℃ or above) is a great challenge [39][172]. However, conversion of cellulosic biomass to ethanol using adapted thermotolerant yeast strains are frequently added at the same time to ensure simultaneous saccharification and fermentation (SSF) was one solution of the above limitations for cost-effective lignocellulosic ethanol production. The SSF operation at high temperatures reduces cooling costs and contamination, and help to minimize enzyme costs, cell death during temperature fluctuations, and inhibition of sugar yield [39][173][174]. Although the temperature between 38 °C and 42 °C tolerate significant reductions in production costs temperature 50 ℃ and above would be more desirable [106]. In this regard, many studies focused on the thermotolerant ethanologenic strains for the SSF of lignocelluloses biomass-based operations, for example, Edgardo et al.,[175] developed a mutant *Saccharomyces cerevisiae* IR2-9a for the SSF of the organosolv-pretreated *Pinus radiata* chips at 40 ℃ with 10 % solids loading in the flask, and the ethanol titer reached 22 g/L; Kadar et al., [176][176] used S. cerevisiae strain for the SSF of the old corrugated cardboard at 40 ℃ at 6 % substrate loadings in the flask, and the maximum ethanol titer was 14.2 g/L; Hari Krishna et al.,[177] used *S. cerevisiae* NRRL-Y-132 for the SSF of the 10% alkaline H_2O_2-pretreated sugar cane leaves at 40 ℃, and the ethanol titer was 18 g/L [178] also reported that high temperature also affects yeast metabolism, as a result, produces secondary metabolites such as glycerol, acetic acid, succinic acid, etc.,[153] . Another study described, *S. cerevisiae* DQ1 strain, using SSF of the dilute acid-pretreated corn stover could tolerate heat up to 40 ℃ and reach maximum ethanol titer of 48 g/L and the ethanol yield of 65.6%. On the other hand, a recent study showed that the maximum level of ethanol was produced at 48 ℃, and the yield was 0.47 g ethanol/g carbohydrate consumed by recombinant *K. marxianus* strain[179]. Although various thermotolerant *S. cerevisiae* strains were tested at 40 ℃ and above, none of them was employed in the SSF at directly at one step SSF conditions. By using both the elevated temperature (52 ℃)

and the high solids loading 30% (w/w) of the pretreated lignocellulose biomass to produce high ethanol titer, which is required by the downstream product purification and reduction in the distillation energy. The newly developed robust and efficient adapted thermotolerant strain *S. cerevisiae* Z100 tolerated up to (52 ºC) and high solids loading and produced 80.1 g/L of ethanol titer, 77.7% of ethanol yield and 0.668 g/L/h of ethanol productivity. The highest productivity so far for cellulosic ethanol production from high solids loading lignocellulosic biomass at high temperature. This was inconsistency with the previous reports on *S. cerevisiae* when the actual lignocellulose was applied under practical operation conditions [176][177][178][175].The present study also provided a possible potential for the future SSCF of lignocellulose feedstock at high temperature and high solid loading to reach high ethanol titer.

3.2 Materials and Methods

3.2.1 Wheat straw and enzymes

Wheat straw was harvested from Dan Cheng, Henan, China in fall 2013 The collected wheat straw was milled coarsely and separated through a mesh with the circle diameter of 10 mm. Then the milled wheat straw was water washed to remove the field dirt, stones, and metal pieces, and air-dried. The composition of wheat straw was determined by the two-step acid hydrolysis method, according to the National Renewable Energy Laboratory (NREL) protocols[156][180]. Commercial cellulase enzyme Cellic CTec 2.0 was kindly provided by Novozymes (China) Investment Co., Beijing, China. The filter paper activity was 203.2 FPU per milliliter of cellulase determined according to the NREL protocol LAP-006 [150]. The cellobiase activity was 4,900 CBU per milliliter of cellulase determined using the method of [151][151]. The total protein concentration was 87.3 mg/ml of cellulase determined by [152][152] method using bovine serum albumin (BSA) as a protein standard. The cellulase enzyme was used grounded on the total protein weight per gram of cellulose substrate in the lignocellulose feedstock. The reagents KH_2PO_4, $(NH4)_2SO_4$, $MgSO_4$, H_2SO_4 were purchased from a local provider Ling Feng Chemical Reagent Co., Shanghai, China. Yeast Extract was from Angel Yeast Co., Yichang, China. Agar was from Aladdin BioChem Co., Shanghai, China.

3.2.2 Strains and media

Biodetoxification fungus *A. resinae* ZN1 was isolated in our previous work and stored in China General Microorganism Collection Center (CGMCC, Beijing, China) with the registration number 7452 [181]. *A. resinae* ZN1 was maintained on potato-dextrose agar (PDA) slant prepared by boiling 200 g of peeled and sliced potatoes in one liter deionized water for 30 min with the addition of 15 g of agar. Ethanol fermentation strain *Saccharomyces cerevisiae* XH7 derived from the wild-type diploid S. cerevisiae strain SF through rationally designed genetic modifications. Then it combined with adaptive evolution in xylose including genomic integration of the novel gene RuxylA encoding xylose isomerase, overexpression of XKS1 encoding endogenous xylulokinase and four genes of non-oxidative pentose phosphate pathway,

and the inactivation of two genes GRE3 and PHO13 encoding aldose reductase and alkaline phosphatase, respectively [182][183]. The strain incubated in YPD medium containing (g/L) 20 of glucose, 20, of peptone, and 10, of yeast extract. The culture vial had deposited in the YPD medium containing 30% of glycerol at −80 °C deep freezer.

The media used included:-

(1) Activation medium containing, 20 g/L of glucose, 20 g/L of peptone, 10 g/L of yeast extract;

(2) seed culture medium, containing, 5% (w/w) of the pretreated and bio-detoxified solid loading wheat straw, without cellulase dosage, 2 g/L of KH_2PO_4, 2 g/L of $(NH4)_2SO_4$, 1 g/L of $MgSO_4$, 10 g/L of yeast extract;

(3) adaptation seed medium, 5% (w/w) of the pretreated and bio-detoxified corn stover, without cellulase dosage, 2 g/L of KH_2PO_4, 2 g/L of $(NH4)_2SO_4$, 1 g/L of $MgSO_4 . 7H_2O$ 10 g/L of yeast extract.

(4) SSCF medium, 2 g/L of KH_2PO_4, 2 g/L of $(NH4)_2SO_4$, 1 g/L of $MgSO_4$, 10 g/L of yeast extract.

The seed culture medium contained an acceptable concentration of xylose from the pretreated wheat straw feedstock and no cellulase addition and no glucose formation throughout the seed culture.

3.2.3 Dry acid pretreatment and biodetoxification

Wheat straw and corn stover were pretreated using the dry acid pretreatment method [154][155][154]. Briefly, 1,200 g of feedstock (dry base) and approximately 500–600 g of 5% (w/w) dilute sulfuric acid solution (conditional on the moisture content of the material) was co-currently fed into the pretreatment reactor for 3 min at the solids/ liquid ratio of 2:1 (w/w). The reactor was 20L in the inner volume and thermally insulated. A single helical ribbon impeller was fixed under the mild agitation rate (50 rpm). The sulfuric acid concentration in the dilute acid solution was maintained in a narrow range allowing to a measured moisture content of the feedstocks. The saturated water steam (1.6 MPa, 201 ℃) was produced from a steam generator machine (HX-36D, Huazheng Boiler Co., Shanghai, China). The pretreatment operation was happening when the hot steam was jetted onto the feedstock bulk in the reactor to175 ±1 ℃ for 5min under the mild helical agitation (50 rpm). Then the pretreated solid feedstocks were released gravitationally from the bottom outlet port. All the dilute acid solution and the condensed water were totally absorbed into the solids to form approximately 50% (w/w) of the dry pretreated biomass solids with the pH around 2.0, and with zero wastewater, the stream was generated. The sulfuric acid in the pretreated biomass solids was neutralized to the pH adjusted at 5.5 by automatic regulation with 5 M NaOH suspension slurry. The pretreated biomass solids were briefly crushed by a disk milling machine (PSB-80JX, Fleck Co., Nantong, Jiangsu, China)

to avoid the extra-long fibers to remove the blockage of pipelines and valves in the downstream flow of the hydrolysate slurry and broth. The pretreated solids were aerobically bio-detoxified in a 15 L bioreactor to remove the inhibitors generated during the dry acid pretreatment operation [154][155]. Briefly, *A. resinae* ZN1 fungus was grown on potato-dextrose agar (PDA) slant at 28 ℃ for sporulation. The spores were collected and diluted to approximately 5–6×10^6 per milliliter of the spore suspension and inoculated onto the pretreated lignocellulose solids at the weight ratio of 10% (the ratio of the spore suspension weight to the pretreated feedstock weight) for five days to the biodetoxification seeds. Then the seed solids were inoculated onto the freshly pretreated solid feedstock at 10% (w/w) inoculation ratio. The biodetoxification was conducted at 28 ℃, and the water-saturated aeration of 0.8 vvm (the ratio of the air input rate in liter per minute to the pretreated biomass volume) for about 30 hrs. The significant inhibitors, including furfural, 5hydroxymethylfurfural (HMF), acetic acid, and phenolic aldehydes, were completely utilized and converted into CO_2 and H_2O and other non-toxic compounds.Similarly the. Xylose and glucose released during the pretreatment were preserved without significant loss because of the strain *A. resinae* ZN1 favours priority for inhibitors as substrates than the sugars. Thus, No cellulose degradation was observed by *A. resinae* ZN1 during the biodetoxification period.

3.2.4 Seed culture preparations from pretreated and bio-detoxified wheat straw

The schematic diagram of the ethanol fermenting adapted thermotolerant strain *S. cerevisiae* Z100 cultured using the freshly pretreated and bio-detoxified wheat straw without cellulase addition used as a carbon source. The adaptation procedure used was to allow the yeast stability for adapting of utilized xylose-containing 15% (w/w) solid feedstock and assure the reproducibility of the fermenting strain. The details are described as follows:

Step-1 A vial of adapted thermotolerant strain *S. cerevisiae* Z100 stock culture was inoculated into a 100 mL flask containing 20 mL of the synthetic medium (20 g/L of glucose, 20 g/L Peptone and 10 g/L yeast extract contained and cultured for 12 h in a shaking incubator at 35 ℃ and 200 rpm.

Step-2 5 mL of the cell culture from step 1 was inoculated into a 250 mL flask with 50 mL of 5% (w/w) freshly pretreated and bio-detoxified wheat straw at 10% (v/v) adaptation nutrient medium and then cultured without cellulase addition for 12 h at 35 ℃ and 200 rpm. In this step, 2.5 grams (dry base) of the pretreated and bio-detoxified wheat straw solids (5% of the total medium by weight percentage) without cellulase and with 10% (v/v) nutrient solutions were added into the medium used for adaptation before the seed culture initiated. (Fig. 3.1a)

Step-3 10 mL of the cell culture from step 2, was inoculated into each 1 Liter flask containing 100 ml nutrient (2 g/L, KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, 1 g/L, $MgSO_4.7H_2O$, 10 g/L, Yeast extract containing medium with solid loading feedstock of 5% (w/w) without cellulase addition were

cultured for 24 h at 35 ^{0}C and 200 rpm In this step, 5 g (dry base) of the pretreated and bio-detoxified wheat straw solids for each flask was added before the seed culture initiated.

Step-4 The operation was continual for further adaptation purposes. That is, 10 ml of the cultured seed from Step 3 was subcultured again into the 100 ml medium used for adaptation and cultured for about 24 hours at 35 ℃ and 200 rpm, and 5 grams (dry base) of corn stover (10%, v/v) without cellulase and 10% (v/v) nutrient solution were added into the medium used for adaptation before the culture initiated.

The cultured cell from Step 4 was used as seed culture of the adapted laboratory evolution processes for the improvement of xylose utilization potential of the newly adapted thermotolerant strain *S. cerevisiae* Z100. (Fig. 3.1b)

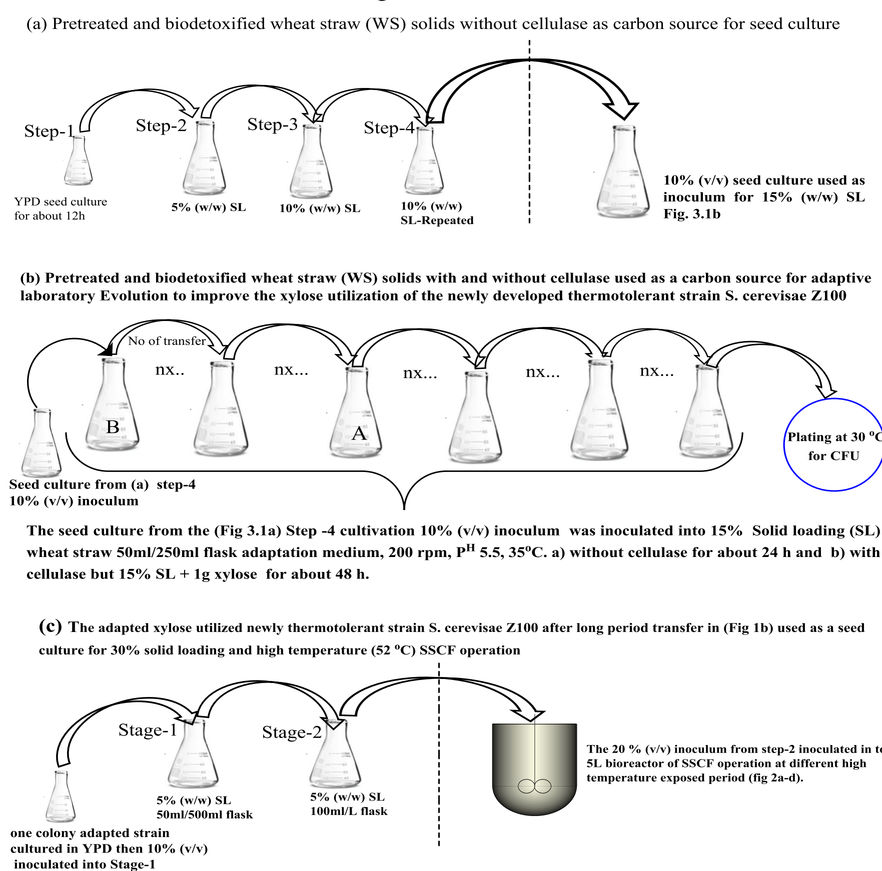


Fig. 3.1 Schematic diagram of Xylose adapted thermotolerant yeast seed culture preparation using freshly pretreated and biodetoxified wheat straw solids without cellulase as a carbon source. Culture condition: 200rpm, 35 ^{0}C, 15% solid loading, pH 5.5 for about 24-48h

3.2.5 Evolutionary adapted of thermotolerant strain *S. cerevisiae* Z100

The xylose utilization capacity of adapted thermotolerant strain *S. cerevisiae* Z100 was evaluated in comparison with the parental *S. cerevisiae strain* XH7 at a higher temperature and 30% solid loading pretreated and bio-detoxified wheat straw as a carbon source. The adapted

thermotolerant strain *S. cerevisiae* Z100 was lost its xylose utilization potential significantly during high temperature fermentation operations. It has became a challenge for the subsequent 30% solid loading wheat straw SSCF operation at high temperature. In this study, we have solved this problem by using adaptive laboratory evolution method based on the following procedures:-

Stage 1. 5 ml of the seed culture from step 4 was inoculated into a 250 ml flask containing 50ml of the adapted medium at 10% (v/v) inoculation ratio, then cultured for 24 h, temp, of 35 ^oC and 200 rpm. In this step, 8gram s (dry base) of pretreated and bio-detoxified wheat straw solids, 15% (w/w) without cellulase was added into the adaptation medium containing nutrients before the culture started.

Stage 2. The adaptive laboratory evolution of the process had been repeated continuously by transferring the cultured yeast cells from the previous medium into the fresh wheat straw solids 15% (w/w) with the same condition. This continuous transfer process was repeated for about 70 days until the xylose utilization performance maintained stable. (Fig. 3.1b)

3.2.6 Simultaneous saccharification and co-fermentation (SSCF)

The simultaneous saccharification and co-fermentation (SSCF) operation was carried out in a 5-L bioreactor with a helical stirring apparatus, as described previously [149]]. The pretreated and bio-detoxified wheat straw and the bioreactor used during SSCF were autoclaved at 121 ℃ for 20 min: Prehydrolysis, 52 ^oC for 4 hrs., pH 5.5, 150 rpm, CTec 2.0 of 15 mg of protein/g of cellulose then add 20% inoculum from adapted seed culture directly inoculate at 52 ^0C (0 hrs heat shock) in 5 L bioreactor containing 30% solids loading feedstock, then off the heat until the temp reaches 30 ^oC manually and then on the heat, and follow the normal operation for about 120 hrs. A similar process has been done with the same fermentation condition but with different heat shock exposing periods. (Fig. 3.2)

From the first up to the fourth SSCF operation each cycles containing the nutrient of 2 g/L of KH_2PO_4, 2 g/L of $(NH_4)_2SO_4$, 1 g/L of $MgSO_4$, and 10 g/L of yeast extract. The samples were centrifuged at 13, 000 g for 5 min and withdrawn at regular intervals, the supernatant was analyzed. The yeast cell viability during SSCF was determined by counting the colony forming units (CFU) on the petridish of the diluted fermenting broth.

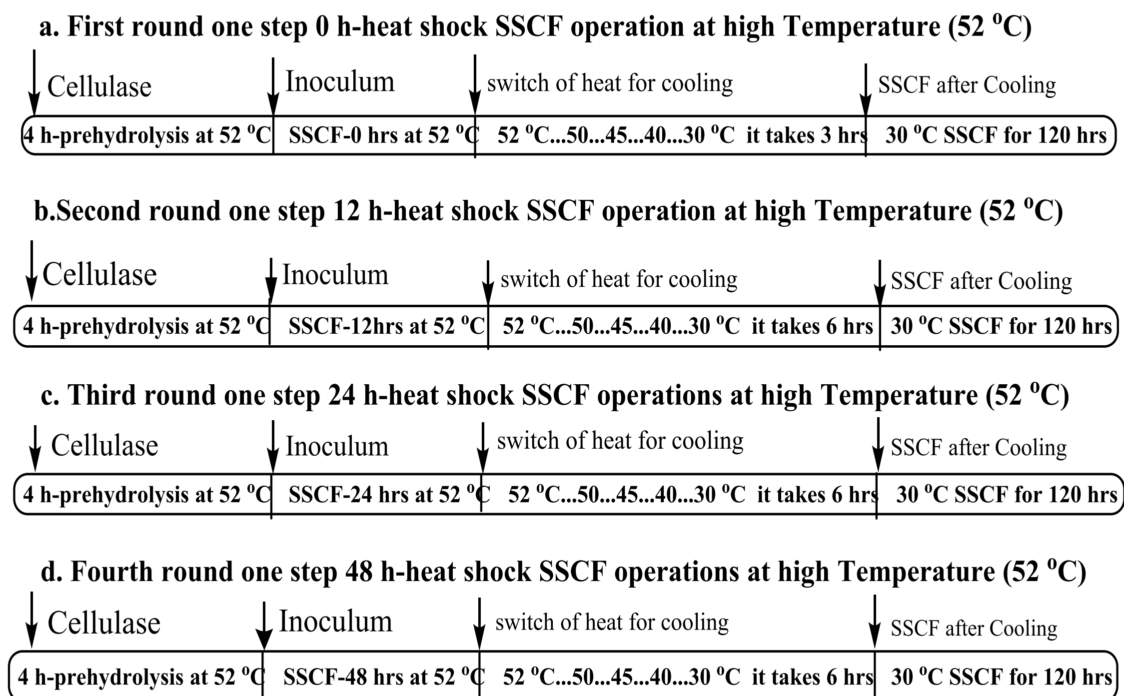


Fig. 3.2 Schematic diagram of the one-step SSCF both enzymatic and SSCF operation processed together at high temperature (52 °C) at a) 0 h at 52 °C b) 12 h at 52 °C c) 24 h at 52 °C and d) 48 h at 52 °C of high temperature. Culture condition: 4h pre hydrolysis, 20% inoculum size, high solid loading (30%), 15 mg of protein/ g of cellulose, pH 5.5, 150 rpm for about 120 h and we used 5M NaOH for pH adjustment.

3.2.7 The temperature condition during SSCF operation

The normal temperature adjustment during pre-hydrolysis and high-temperature cellulosic ethanol SSCF operation in our practical study was 50 °C but when we used the internal heat measuring thermometer during SSCF operation, the real temperature at the time of fermentation operation inside the 5 L bioreactor became 52 °C these were confirmed at 0, 12, 24 and 48 hrs long heat exposed period. The cooling process for each period of fermentation took place without the usage of water until it reached 30 °C. The overall operation was stated (Fig. 3.2a-d) and the cooling time of the bioreactor for each operation was carefully recorded for further analysis.

3.2.8 Analytical methods

Glucose, ethanol, and lignocellulose degradation, such as furfural, 5-hydroxy methyl furfural, and acetic acid, were measured using an HPLC (LC-20 AD, refractive index detector RID-10A, Shimadzu, Kyoto, Japan) equipped with a Bio-rad Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA) at the column temperature of 65 ℃ with 5 mM H_2SO_4 as eluent and with a flow rate of 0.6 mL/min. Samples periodically taken from the fermentations

were centrifuged at 13,000 rpm for 5 min and then filtered done by the 0.22-μm filter before injection.

Ethanol yield was calculated according to the method described by [160].

$$\text{Ethanol yield} = \frac{[C_1 \times W]}{976.9 - 0.804 \times [C_1]} \times \frac{1}{0.511 \times f \times [Biomass] \times m \times 1.111} \times 100\%$$

where $[C_1]$ is the ethanol concentration in the culture broth (g/L), W is the total water input of the SSF (g), f is the cellulose fraction of corn stover feedstock, [Biomass] is the dry Wheat straw concentration at the initial stage of SSF operation (g/g), m is the total weight of the SSF (g), 0.511 is the conversion factor for glucose to ethanol based on stoichiometric biochemistry of yeast, 1.111 is the conversion factor for cellulose equivalent to glucose, and 976.9 and 0.804 are the constants in previous study developed by Zhang and Bao [160]

3.3 Results and Discussion

3.3.1 Seed culture preparation and cell growth condition at high temperature

The cell growth of newly adapted thermotolerant and xylose utilizing strain *S. cerevisiae* Z100 was significantly higher than that of parental strain *S. cerevisiae* XH7 as cell counting by CFU for each high temperature (52 ºC) exposing periods of (0, 12, 24 and 48 hrs) (Fig. 3.5a). The detailed cell growth condition as follows; the final cell culture at the 70th days repeated transfer every 24 hrs was taken for plating at 30 ºC for about 48 hrs, then a single colony from the previous culture was transferred into a 20 ml/100 ml flask containing, YPD media and incubated for about 12 hrs (Fig. 3.1c). Then 5 ml inoculum from the first was transferred into 5% solid loading wheat straw containing medium and cultured for about 12h and 24 hrs without cellulase enzyme at 35 ºC, 150 rpm, pH 5.5 (Fig. 3.3). The two strains showed almost the same cell growth performance after 12 h and 24 h seed culture respectively. Then the 12 h seed culture was used for the next SSCF operations throughout this experiment.(Fig. 3.3a).

Consequently, during SSCF operation at high solid loading and temperature the decreased cell growth in the parental strain *S. cerevisiae* XH7 under higher levels heat exposure periods observed at (0, 12, 24 and 48 hrs.) (Fig. 3.5a), previous study indicated that the decrease in cell growth might be accelerated by cell wall decomposition and the reduction of cell growth by cellulase, [153][184][185]. A similar study also confirmed that the decrease in cell growth in the SSF of the dilute acid-pretreated corn stover due to the increase of substrate or cellulase loadings at the elevated temperatures [153]. While, the newly adapted thermotolerant strain *S. cerevisiae* Z100 at higher temperature was attributed to the success and stability of both improved cell

growth and successful adaptability of xylose utilization for all similar heat exposed periods (Fig. 3.5a; Fig. 3.6)

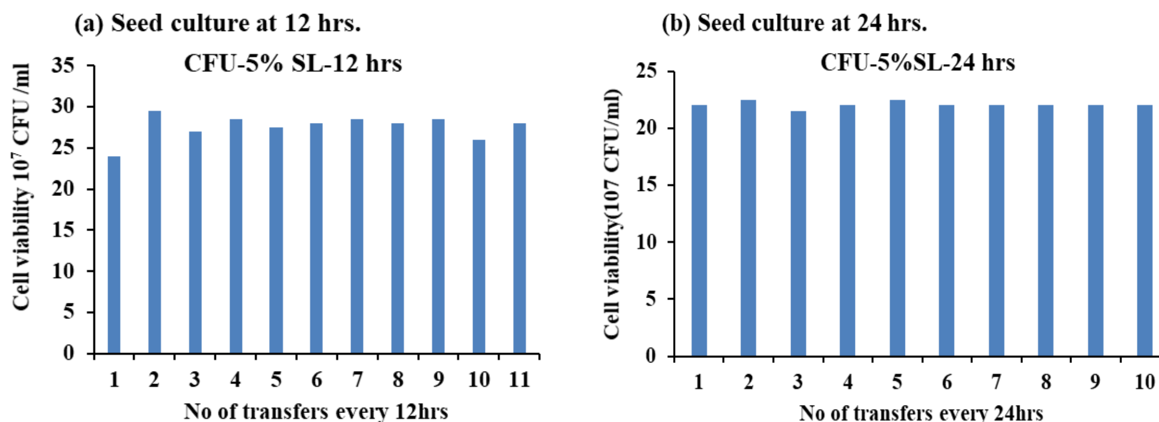


Fig 3.3 Seed culture time optimization taken from the 70th transfer adapted seed culture used as an inoculum for SSCF operation (a) Seed culture preadaptation before SSCF operation using 5% (w/w) solid loading without cellulase for about 12 h. (b) Seed culture preadaptation before SSCF operation using 5% (w/w) solid loading without cellulase for about 24 h at 200 rpm, pH 5.5, 35 ºC , using 50 ml/500 ml flask with the same seed culture condition.

3.3.2 Adaptive laboratory evolution for xylose utilization

The parental strain before heat stress adaptation had consumed the xylose significantly but after adaptation of heat stress the newly adapted thermotolerant strain *S. cerevisiae* Z100 was unable to utilized xylose significantly, for each exposed heat shock period (Fig 3.1a-d). The practical study was focused on solving this major challenge by applying evolutionary adaptation method. The operations were conducted by the following two approaches:- with cellulase and without cellulase. The 15% (w/w) solid loading wheat straw feedstock with cellulase and 10% inoculum was cultured at 35 ºC, 200 rpm using flask fermentation for about 48 hrs, the result showed that, the xylose utilization was very poor, but the cell growth has been acceptable condition, this may be due to the glucose inhibition effect on the xylose utilization. On the second approach, the 5ml seed culture from the first was subcultured into the second flask containing the same seed culture medium but without cellulase for about 70 days, the result showed that long period of transfer without cellulase showed better xylose utilization potential than the first (B) (Fig. 3.4a) this improvement might be due to the absence of the cellulase enzyme results in poor glucose formation then the strain forced to utilized xylose and long period of adaptability at this situation made the strain stable (Fig. 3.4a). The detailed procedure of adaptation described in the material and methods. In general, the improved xylose utilization was due to the improvement of the cell CFU number ($3.0x10^8$) in the first (B) was reached a maximum of (48 hrs.) culture time with cellulase but poor xylose utilization. In the

second (A) the CFU number was almost similar to the first and consumed the xylose, approximately 90% of the initial xylose concentrations (Fig. 3.4a) within 24 hrs. Moreover, overall cell growth and xylose utilization in the second cultures (A) was much better than the first and met the requirement of the next SSCF operation. Similar xylose utilization potential ratio of the two strains has been evaluated during high solid loading and high temperature SSCF operations.(Fig. 3.6) The previous study indicated that the adaptive evolution method contributed to the reduction of the seed culture cost 错误!未找到引用源。, similarly, unlike the previous research which was done by [186], in this practical study, also improved the two-step seed culturing method without cellulase addition (Fig. 3.1). In general, newly strain *S. cerevisiae* Z100 surpassed the parental XH7 both in xylose utilization and ethanol production during SSCF operation at similar condition. (Fig. 3.5).

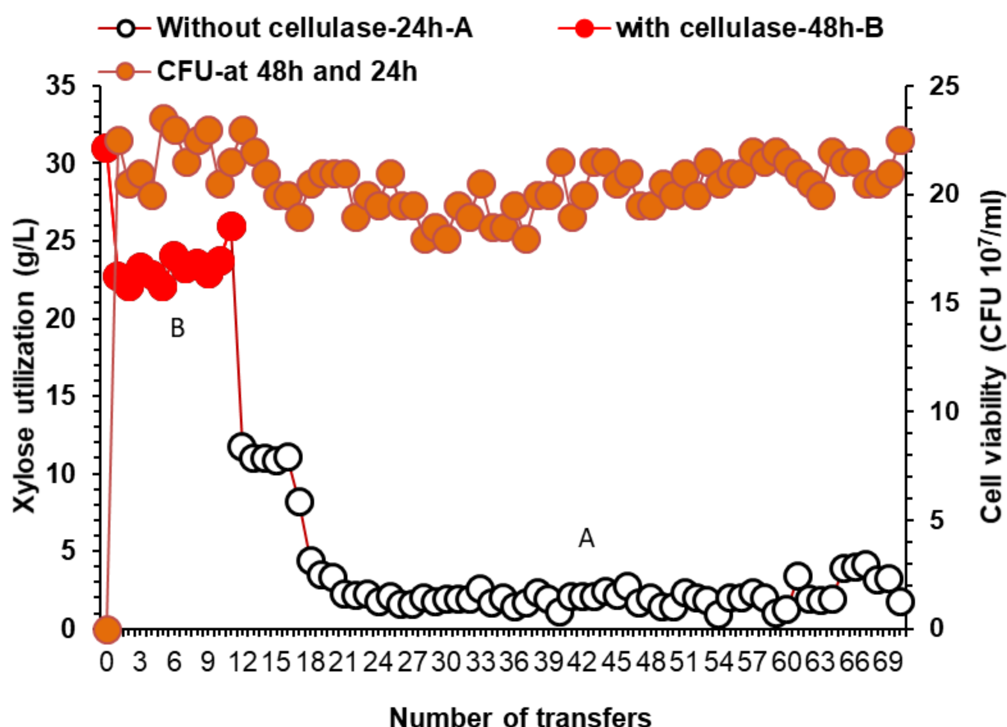
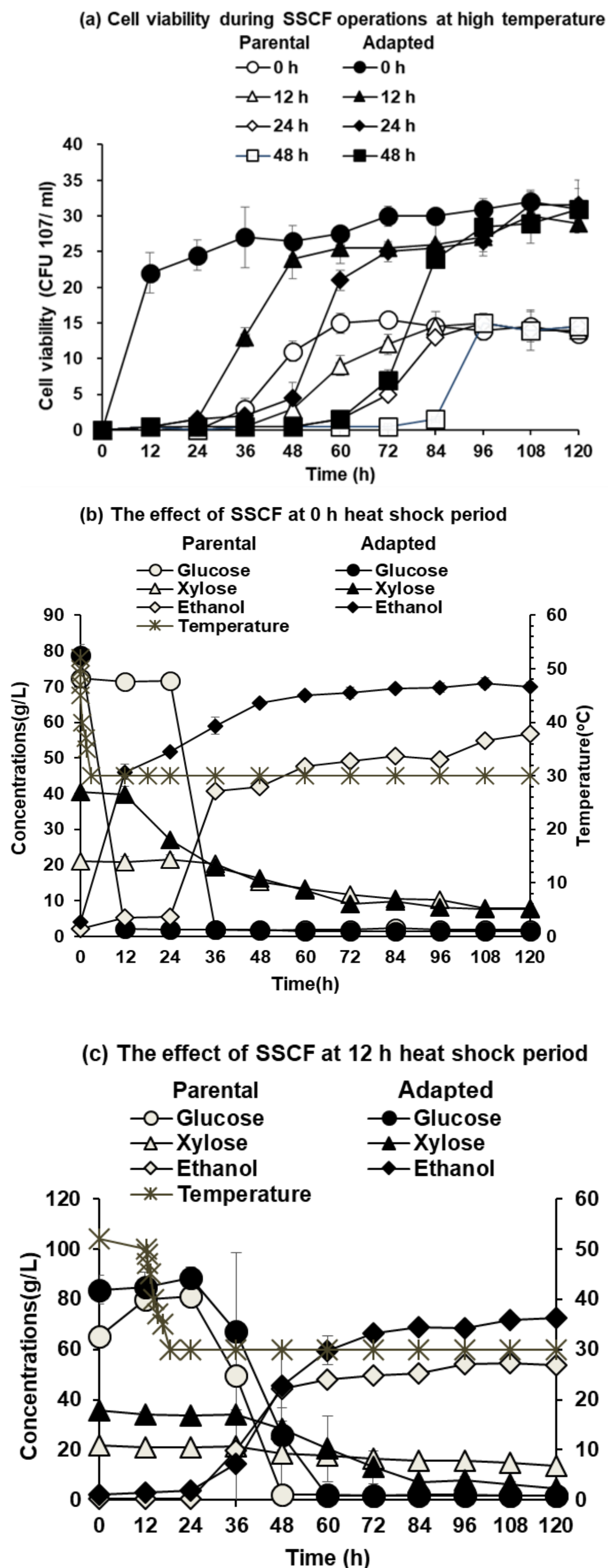


Fig. 3.4 Xylose adaptation using 15% (w/w) solid loading (A) without cellulase for about 24h and (B) with cellulase for about 48 hrs. then continuous transfer 10% of Inoculum from (B) into (A) seed culture medium containing freshly pretreated and bio-detoxified wheat straw at 35 °C, 200 rpm, pH 5.5, 50 ml/250 ml flask every 24 hrs. for about 70 days until the xylose utilization performance of the strain in (A) was successively maintained stable.

3.3.3 Cellulosic ethanol production at high temperature using (SSCF) operation

The SSCF fermentation of pretreated and bio-detoxified lignocellulose wheat straw feedstock 30% (w/w) solid loading at high temperature (52 ºC) were pre-hydrolyzed in a short period (4 hrs.) at 52 ℃, pH 5.5, 15 mg of proteins/g of cellulose, 20% inoculum 150 rpm for about 120 hrs, at different high temperature exposing periods 0, 12, 24 and 48 hrs in the specially designed 5L bioreactors equipped with helical ribbon impeller [14]. (Fig. 3.2). And, pH was adjusted 5,5 throughout the fermentation processes by adding 5 M NaOH solution to lessen the adsorption of cellulase on lignin [187]-[190]. The final ethanol titer of each exposed period at (0, 12, 24 and 48 hrs.) produced 70, 73, 73, 80.1 g/L and 56.7, 53.8, 33.2, and 40.4 g/L after 120 hrs fermentations by adapted thermotolerant strain Z100 and parental strain XH7, respectively. The cell growth of adapted strain Z100 was significantly higher than that of parental strain XH7 as measured by CFU for all heat exposed periods (Fig. 3.5a; Fig. 3.7a). However, parental strain at high temperature produced lactic acid of 2.07, 3.43, 29.7 and 17.5 g/L at different heat exposed periods of 0, 12, 24, and 48 h respectively. (Fig. 3.7b). This may be due to the strain sensitivity and poor tolerance on high temperature. Furthermore, both the adaptive and the parental strains cell growth used much time in lag phase during the high temperature stress conditions and for each SSCF operation at high temperature the cooling time from 52 ºC to 30 ºC last at least (6 hrs.) in addition to the exposed periods except 0 h heat shock (Fig. 3.2a) this may also due to the immediate addition of the inoculum and switching off the heat directly contributed to shortening the cooling time of the bioreactor. Consequently, the ethanol production increased with the increase of the heat-shocked periods at 70.1 g/L for 0 h heat shocked and highest 80.1 g/L of the ethanol titer obtained after 48 h heat shocking period. The increased portion of ethanol production was attributed to the thermotolerance of the adapted strain, due to direct utilization of glucose and xylose and converted into ethanol. (Fig. 3.5e; Table 1). The parental strain *S. cerevisiae* XH7 ethanol titer, Yield, and productivity were lower for each long period of heat exposure compared with that of the adapted thermotolerant strain *S. cerevisiae* Z100 in all high-temperature heat shocking period. Consequently, the above finding was inconsistent with the previous study done by [153] indicated that ethanol production lowered at elevated temperature using 25% solid loading and almost halted at 30% solid loading. Thus, newly adapted strain *S. cerevisiae* Z100 provided a practical potential for the future SSCF lignocellulose feedstock at high temperature and solid loading to reach high ethanol titer.


(a) Cell viability during SSCF operations at high temperature
Parental
Adapted
0 h
12 h
24 h
48 h
Cell viability (CFU 107/ ml)
Time (h)
(b) The effect of SSCF at 0 h heat shock period
Parental
Adapted
Glucose
Xylose
Ethanol
Temperature
Concentrations(g/L)
Temperature(°C)
Time(h)
(c) The effect of SSCF at 12 h heat shock period
Parental
Adapted
Glucose
Xylose
Ethanol
Temperature
Concentrations(g/L)
Temperature (°C)
Time (h)

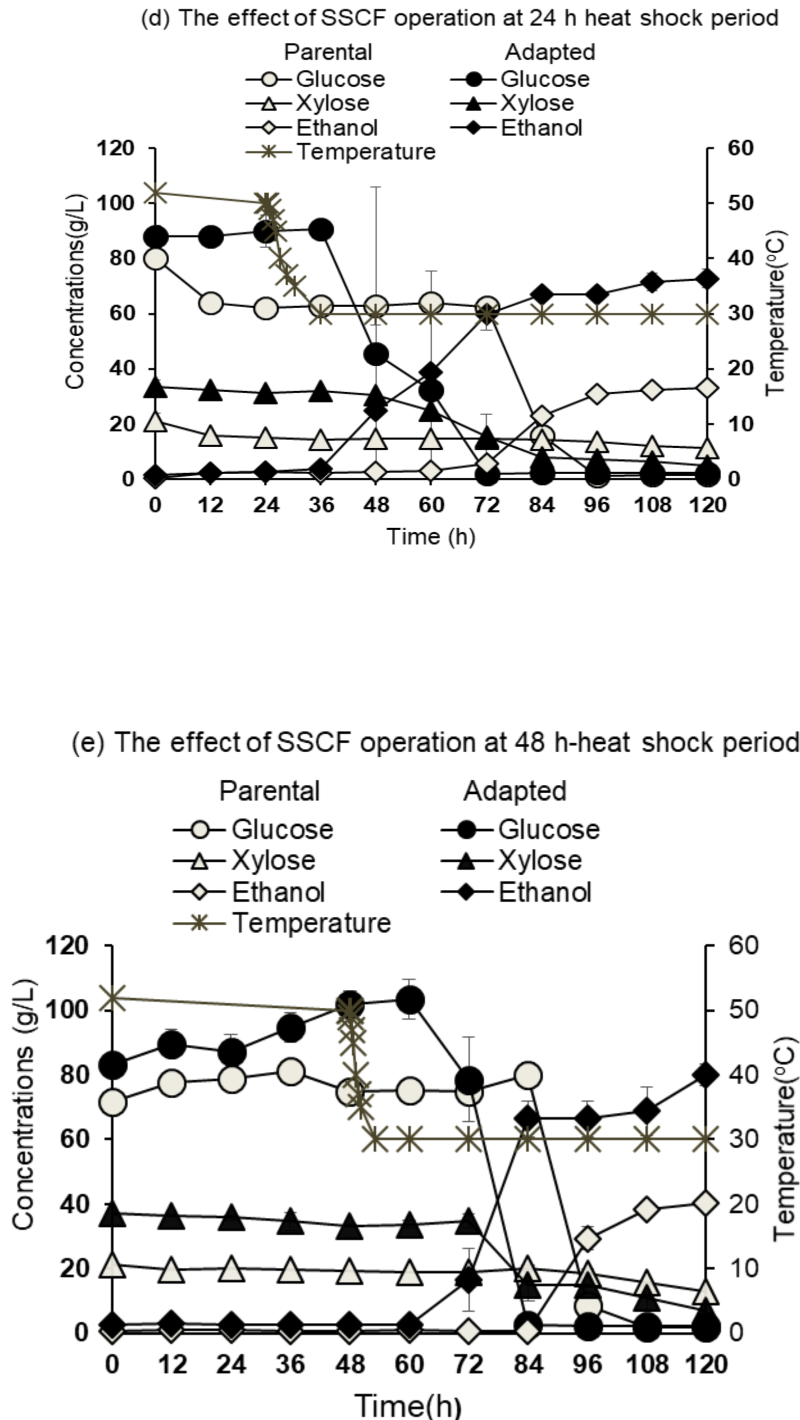


Fig. 3.5 Comparison of ethanol fermentation performance between adapted strain *S. cerevisiae* Z100 and parental strain *S.cerevisae* XH7 from solid wheat straw prepared at different heat exposed periods at 0, 12, 24, and 48 hrs. (a) Cell growth response as measured by CFU; (b-e) ethanol production comparison between the two strains under the same conditions using the wheat straw during SSCF Fermentation conditions: 30% (w/w) solid loading 52 ℃, pH 5.5, 15 mg of protein/g of cellulose, 20% inoculum, 150 rpm at the heat exposed periods of 0, 12, 24 and 48 hrs., respectively in newly adapted strain *S. cerevisiae* Z100 (Black) and Parental strain *S. cerevisiae* XH7 (White)

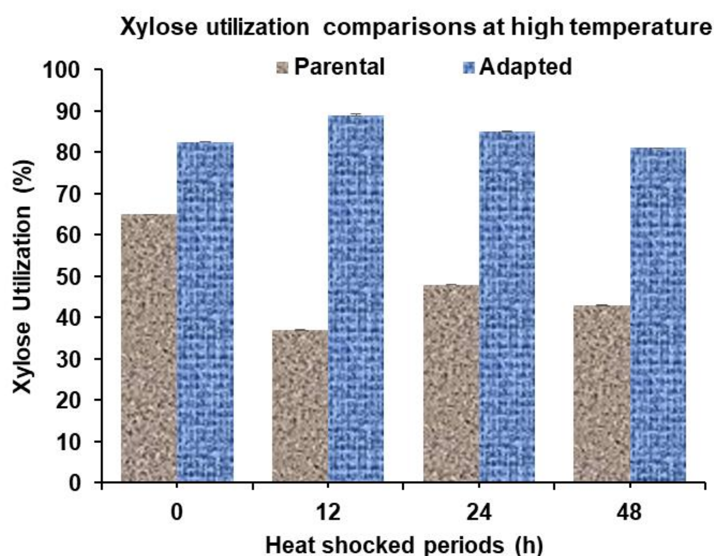
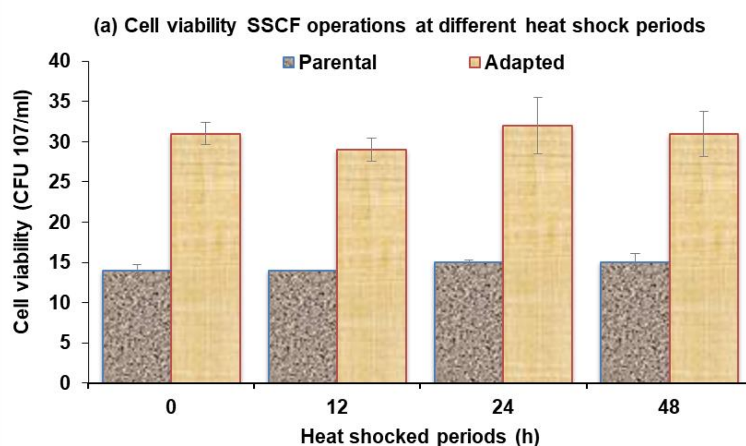


Fig. 3.6 Xylose utilization comparisons at high temperature SSCF conditions (Gray) parental strain XH7,(Blue) Adapted strain Z100, using the wheat straw during SSCF Fermentation conditions: 30% (w/w) solid loading 52 ℃, pH 5.5, 15 mg of protein/g of cellulose, 20% inoculum, 150 rpm at the heat exposed periods of 0, 12, 24 and 48 hrs.

3.3.4 Evaluation of cellulosic ethanol production potentials

The thermo-tolerance and the growth potential of strain *S. cerevisiae* Z100 and parental strain *S .cerevisiae* XH7 were examined in the 5% (w/w) solid medium at 35 ºC, throughout the operation, as shown in (Fig. 3a.) at the same time the ethanol production potential and Cell growth by CFU at high temperature using SSCF operation for long periods (0, 12, 24, and 48 hrs) were also evaluated in this study. In our practical study, a sharp cell growth improvement had occurred after some time when the temperature was reduced to 30 ºC without the usage of water for cooling in both strains with significant growth difference (Fig. 3.5a; Fig. 3.7a). A study conducted by [178] reported that high temperature also affects yeast metabolism as a result produces secondary metabolites such as glycerol, acetic acid, succinic acid, etc. Consistent with the previous study the production of glycerol by adapted thermotolerant *S. cerevisiae* Z100 at high temperature increased in increasing temperature and produced, 9.96, 10.13, 10.69 and 11.36 g/L of glycerol for each exposed periods. While non-adapted parental strain *S. cerevisiae* XH7 produced 4.2, 5.43, 5.7 and 5.81 g/L for each shocking heat period of (0, 12, 24, and 48 hrs.), respectively (Fig. 3.7c) and (Table 3.1), however, the acetic acid formation during the two strain fermentations at similar conditions was almost identical in both strains (Fig. 3.7d). In general, in the past, many researchers focused on the thermotolerant ethanologenic strains for the SSF of lignocelluloses biomass-based operations; for example, Edgardo et al., [175]developed a mutant *Saccharomyces cerevisiae* IR2-9a for the SSF of the organosolv-pretreated Pinus radiata chips at 40 ℃ with 10 % solids loading in the flask, and the ethanol

titer reached 22 g/L; Kadar et al., [176] used an *S. cerevisiae* strain for the SSF of the old corrugated cardboard at 40 ℃ at 6 % substrate loadings in the flask, and the maximum ethanol titer was 14.2 g/L; Hari Krishna et al., [177] used *S. cerevisiae* NRRL-Y-132 for the SSF of the 10 % alkaline H_2O_2-pretreated sugar cane leaves at 40 ℃, and the ethanol titer was 18 g/L, *S. cerevisiae* DQ1 strain, using SSF of the dilute acid-pretreated corn stover could tolerate heat up to 40 ℃ and reach maximum ethanol titer of 48 g/L and the ethanol yield of 65.6 % [178]and recent study showed that the maximum level of ethanol produced at 48 ℃, and the yield was 0.47 g ethanol/g carbohydrate consumed by recombinant *K. marxianus* strain [179]although various thermotolerant *S. cerevisiae* strain tested at 40 ℃ and above, none of them was employed in the SSCF at directly at one pot fermentation including both the elevated temperature (52 ℃) and the high solids loading of the pretreated lignocellulose biomass to produce high ethanol titer, which is required by the downstream product purification and reduction in the distillation energy. In this practical study, however, we developed the robust and efficiently adapted thermotolerant strain *S. cerervisiae* Z100 that tolerated up to (52 ºC) and produced 80.1 g/l, of ethanol titer, 77.7%, of yield and 0.668 g/L/h of productivity after 120 hrs fermentation operations,(Fig. 3.5e) demonstrated that, this newly strain outshined the previously studied ones significantly and showed better fermentation performance than the previously reported strains and other yeasts, when the actual lignocellulose operation applied under practical operation conditions [175][176][191] In addition, the comparison result of the different biochemical during high-temperature SSCF operations was summarized in (Table 3.1). Furthermore, the observable advantages of the present work were demonstrated not only at the high ethanol titer, yield, and productivity, but also the significant reduction of wastewater generation, avoiding water utilization for cooling, and cell death during temperature fluctuation and providing one pot fermentation technology and improvement of cellulase activities for potential cost reduction.



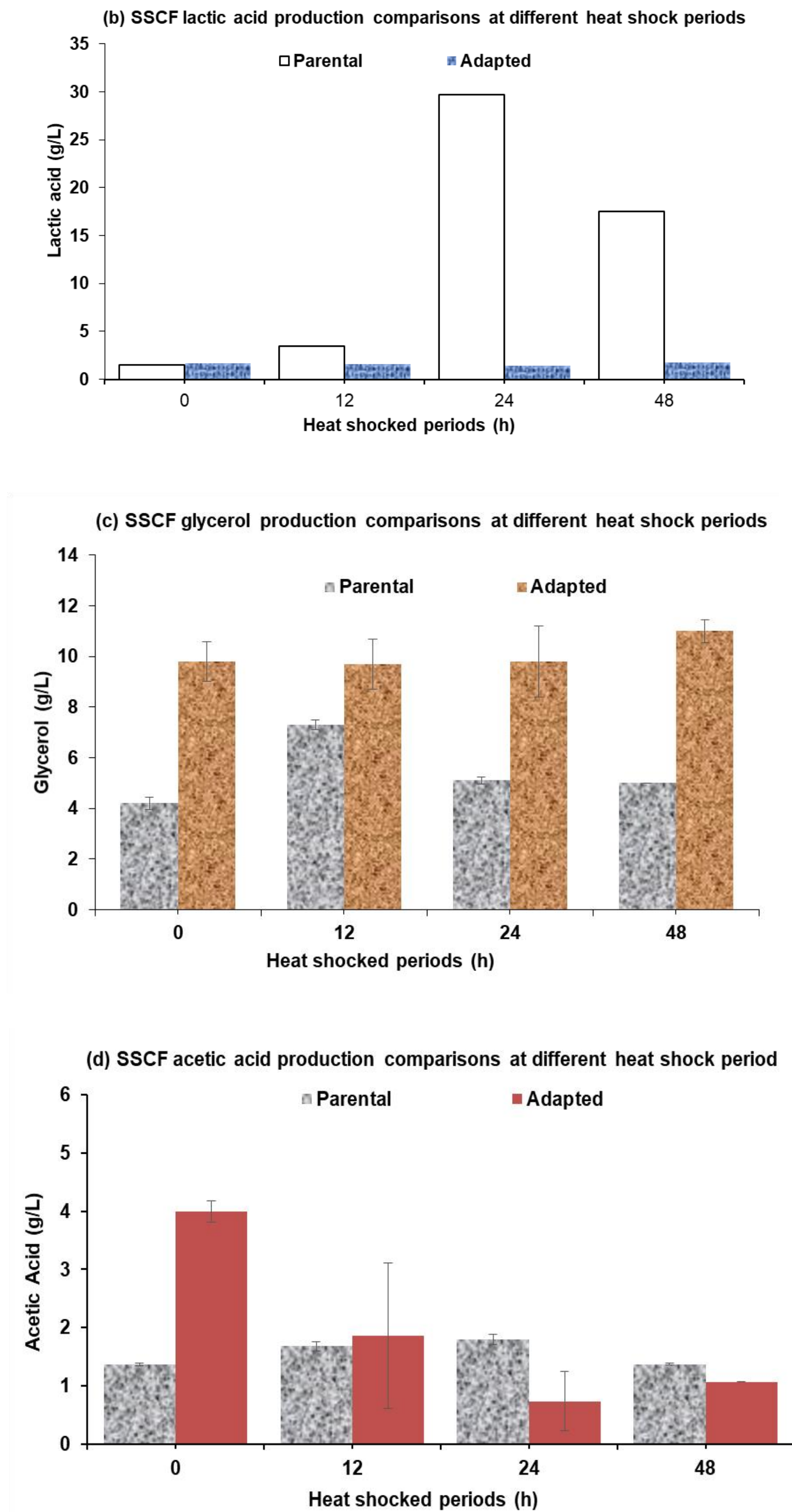


Fig. 3.7 The lactic acid, glycerol, and acetic acid production comparisons between adapted thermotolerant strain Z100 and parental strain *S.cerevisae* XH7 (a) Cell viability comparison during high temperature SSCF operations (b) lactic acid production (c) glycerol production and (d) acetic acid production using SSCF operations at high solid loading (30%)

and high temperature of 52 ºC, exposed period of (0, 12, 24 and 48 hrs) 15 mg/g of cellulose, pH 5.5, 150 rpm, 20% (v/v) inoculum for about 120 hrs.

Table 3.1 Comparison result between Parental strain *S.cerevisiae* XH7 and newly adapted strain *S. cerevisiae* Z100 during 30% solid loading 5 L bioreactor at high-temperature SSCF operation for about 120 hrs.

Strains	Heat shock (hrs)	Ethanol (g/L)	Yield (%)	Productivity (g/L/h)	Glycerol (g/L)	Lactic acid (g/L)	Acetic acid (g/L)
Parental	0	56.7	53.99	0.473	4.20	2.07	1.37
Adapted	0	70.1	66.4	0.584	9.96	0.92	3.99
Parental	12	53.8	51.12	0.448	5.43	3.43	1.86
Adapted	12	72.8	69.89	0.606	10.13	1.1	1.86
Parental	24	33.2	30.97	0.277	5.70	29.71	1.79
Adapted	24	72.8	70.33	0.606	10.69	1.09	0.73
Parental	48	40.4	37.95	0.337	5.81	17.53	1.37
Adapted	48	80.1	77.7	0.668	11.36	1.16	1.07

3.4 Conclusion

In the first practical study in this thesis, we reduced the cellulase enzyme dosage using the β-glucosidase enzyme producing strain *Clavipora* NRRL Y-50464 at high solid loading. In this practical study, ethanol titer of 38.1 g/L and conversion efficiency of 55.5% were obtained from 25% solids loading (w/w) with a low cellulase dose of 5 mg protein/g glucan without the addition of extra β-glucosidase. The robust native β-glucosidase activity generated from *Clavispora* NRRL Y-50464 provided sufficient complementary hydrolysis capability to reduce the enzyme cost for cellulosic ethanol production by SSF. On the second practical study we also developed newly thermotolerant strain *S. cerevisiae* Z100. The successful adaptation of high temperature and xylose utilization in this study was the greatest achievement in our biorefinery laboratory adapted Z100 was operated at one-step SSCF at high temperature, and solid loading 30 % produced 80.1 g/L of ethanol titer, 77.7% of ethanol yield, and 0.668 g/L/h ethanol productivity after. 120 hrs fermentation operations. While the parental strain *S. cerevisiae* XH7 produced 40.4 g/L, of ethanol titer, 37.95% of ethanol Yield and 0.337 g/L/h of ethanol productivity at similar SSCF operations for about 120hrs. Moreover, the newly adapted thermotolerant strain *S. cerevisiae* Z100 showed a strong thermotolerance and potentials of a significant amount of xylose utilization in one-step regular SSCF operations. The one-pot fermentation at elevated temperature (52 ºC) using adapted thermotolerant strain *S. cerevisiae* Z100 tolerated the greater cellulase dosage and solids loading of the pretreated wheat straw and resulted in increased ethanol production. Thus, the practical study provided a real potential for the future SSCF using newly adapted thermotolerant and xylose utilized strain *S. cerevisiae* Z100 at a high solid loading of lignocellulose feedstock and high temperature to reach high

ethanol titer. Furthermore, the observable advantages of the present work were demonstrated not only at the high ethanol titer, yield, and productivity, but also a significant reduction of zero level wastewater generation, zero level water utilization for cooling, zero levels cell death during temperature fluctuation and providing one-pot fermentation technology for potential cost reduction.

Chapter 4

General conclusion and future perspective

The objectives of the present practical study were to reduce the lignocellulosic processing and operational cost for making the lignocellulosic biorefinery economically feasible and environmentally sustainable to produce ethanol and other value-added chemicals like glycerol. In this study, several strategies were developed and evaluated by improving the ethanol titer and yield by reducing the cellulase dosage and using innate β-glucosidase producing strains *Claviopra* NRRL Y-50464 for cost reduction (Chapter 2). The following are merits of Strain *Clavispora* NRRL Y-50464

1. Exhibited a superior ethanol fermentation performance over *Saccharomyces cerevisiae* DQ1 under similar conditions. It produced the ethanol titer of 38.1 g/L within 96 h at a conversion efficiency of 55.5% with 25% solid loading (w/w) via SSF without the addition of the extra β-glucosidase supplement.
2. Improved performance of Y-50464 using a bioreactor with a helical stirring apparatus confirmed its benefit over the conventional bioreactors initially designed for liquid fermentations in cellulosic ethanol transformation by SSF.
3. The results of this study suggested that the strain *Clavispora* NRRL Y-50464 has a potential candidate for lower-cost cellulosic ethanol production from lignocellulosic feedstock.

   However, the strain Y-50464 has poor glucose metabolism activity compared to DQ1 for ethanol production so it needs further adaptation or genetic engineering study.

The other finding in this thesis is that the use of high-temperature tolerant strain *S. cerevisiae* Z100 and use of the adaptive method for xylose utilization improvement described in (Chapter 3), This SSCF fermentation improves the following:-

1. It improves the ethanol yield significantly and reduces the cost for yeast seeds culture preparation by using freshly pretreated solid lignocellulosic material as a carbon source without cellulase addition (the new method). And
2. The practical result using newly developed thermotolerant strain S.cerevisae XH7 showed that the strain was produced high ethanol titer (80g/l), ethanol yield (77.7%) and ethanol productivity (0.668 g/L/h) at higher temperature SSCF operations.
3. The adapted thermotolerant xylose-utilizing strain *S. cerevisiae* XH7 and using freshly pretreated and bio-detoxified feedstock without cellulase and glucose as a carbon source for preparation of yeast seeds culture reduced the cost significantly,
4. Reduction of water usage for cooling the bioreactor during the SSCF operations, this helps the reduction on energy cost, contamination, and cell death during temperature fluctuations and

encourage one pot fermentation.

However the adapted strain still needs further study for including detailed molecular gene sequencing which of course part of this study and it is ongoing processes. Furthermore, the seed culture adaptation without cellulase should be further confirmed it's potential by using different solid feedstock. In general the current practical study, we developed thermotolerant and xylose utilized potential strain S. cerevisiae Z100 and the study showed significant result improvement of cellulosic ethanol production. Thus, in the future we have to check its stability and productivity with different feedstock, further work on the train to make inhibitor and low pH tolerant using adaptive laboratory evolution or metabolic engineering for commercial scale cellulosic ethanol industry.

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- Geberekidan, M., Zhang, J., Liu, Z., and Bao J., Improved cellulosic ethanol production from corn stover with a low cellulase input using a β-glucosidase-producing yeast following a dry biorefining process. Bioprocess and Biosystems Engineering, 2018. 018, 2034-9
- Geberekidan, M., Zhao, Y., Zhang, J., and Bao, J., Ethanol fermentation from wheat straw at high temperature using thermotolerant and xylose utilized adapted strain *S. cerevisiae* Z100 - on the processes for submission

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