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Research review paper

Development of yeast cell factories for consolidated bioprocessing of lignocellulose to bioethanol through cell surface engineering

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ABSTRACT

To build an energy and material secure future, a next generation of renewable fuels produced from lignocellulosic biomass is required. Although lignocellulosic biomass, which represents an abundant, inexpensive and renewable source for bioethanol production, is of great interest as a feedstock, the complicated ethanol production processes involved make the cost of producing bioethanol from it higher compared to corn starch and cane juice. Therefore, consolidated bioprocessing (CBP), which combines enzyme production, saccharification and fermentation in a single step, has gained increased recognition as a potential bioethanol production system. CBP requires a highly engineered microorganism developed for several different process-specific characteristics. The dominant strategy for engineering a CBP biocatalyst is to express multiple components of a cellulolytic system from either fungi or bacteria in the yeast *Saccharomyces cerevisiae*. The development of recombinant yeast strains displaying cellulases and hemicellulases on the cell surface represents significant progress toward realization of CBP. Regardless of the process used for biomass hydrolysis, CBP-enabling microorganisms encounter a variety of toxic compounds produced during biomass pretreatment that inhibit microbial growth and ethanol yield. Systems biology approaches including disruptome screening, transcriptomics, and metabolomics have been recently exploited to gain insight into the molecular and genetic traits involved in tolerance and adaptation to the fermentation inhibitors. In this review, we focus on recent advances in development of yeast strains with both the ability to directly convert lignocellulosic material to ethanol and tolerance in the harsh environments containing toxic compounds in the presence of ethanol.

Contents

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1. Introduction

Utilization of biomass as the starting material for various chemicals and for the production of fuels has received considerable interest in recent years. Environmental concerns and the depletion of oil reserves have resulted in governmental actions and incentives to establish greater energy independence by promoting research on environmentally benign and sustainable biofuels. Bioethanol is currently one of the most promising alternatives to conventional transport fuels because of its desirable characteristics such as high octane value and good combustion efficiency. Also, the use of ethanol produced from biomass as a transport fuel can help in reducing CO_2 buildup by recycling CO_2 that is released when bioethanol is combusted as fuel.

Polysaccharides present in lignocellulosic materials such as sugarcane bagasse, corn stover, wheat straw, grasses, wood chips and other agricultural residues, are of great interest as feedstocks for second-generation ethanol production, because they are abundant, inexpensive and renewable sources of sugar. However, the processes involved are more complicated than it from the sources such as cornstarch and cane juice, which means the costs of ethanol production from lignocellulosic materials are higher. Ethanol production from lignocellulosic materials includes the following main steps: (1) chemical and physicochemical pretreatment to swell the biomass, (2) hydrolysis of cellulose and hemicellulose to fermentable sugars by enzymes such as cellulases and hemicellulases, (3) microbial fermentation for the production of ethanol, (4) separation and concentration of ethanol by distillation and dehydration (Sánchez and Cardona, 2008). Therefore, consolidated bioprocessing (CBP), which combines enzyme production, saccharification of cellulose and hemicellulose, and fermentation into a single process, is increasingly recognized as having potential for low-cost production of ethanol, since the costs of capital investment, substance and other raw materials, and utilities associated with microbial enzyme production can be avoided (Cardona and Sánchez, 2007; Lynd et al., 2005; Xu et al., 2009). However, natural microorganisms with the capability for efficient enzyme production, lignocellulose saccharification, and ethanol production are not currently available. Therefore, CBP requires a highly engineered microbial workhorse developed for several different process-specific characteristics (Fig. 1).

Development of such an organism has been pursued by two strategies, native and recombinant cellulolytic strategies (Lynd et al., 2005). The native cellulolytic strategy involves engineering naturally cellulolytic microorganisms to improve ethanol production. Some thermophilic anaerobes belonging to the genera *Clostridium* were the first candidates (Demain et al., 2005; Jin et al., 2011; Lynd et al., 2002; Xu et al., 2010). *Clostridium thermocellum* displayed one of the fastest growth rates on crystalline cellulose by use of large enzyme complexes, termed cellulosome (Lynd et al., 2002), which has been regarded as one potential CBP-organism. Some fungi, such as *Aspergillus*, *Fusarium*, *Monilia*, *Neocallimastix*, *Rhyzopus* and *Tricoderma* have been reported to possess the ability to convert cellulose to ethanol (Anasontzis et al., 2011; Gong et al., 1981; Panagiotou et al., 2005; Ruiz et al., 2007; Skory et al., 1997; Stevenson and Weimer, 2002). In particular, *Trichoderma reesei* strains secreted larger quantities of saccharolytic enzymes, which can be considered as a candidate for CBP (Xu et al., 2009). The crucial research objectives for this strategy include increasing the ethanol titer, eliminating byproducts, and improving the tolerance to ethanol. The recombinant cellulolytic strategy involves engineering non-cellulolytic organisms that exhibit high ethanol yield and titer so that they express a heterologous cellulase system to enable cellulose utilization. Heterologous expression of cellulases and hemicellulases has been primarily studied in bacterial hosts such as engineered *Escherichia coli* (Ryu and Karim, 2011), *Klebsiella oxytoca* (Zhou and Ingram, 2001; Zhou et al., 2001) and *Zymomonas mobilis* (Linger et al., 2010; Vasan et al., 2011) and the yeasts *Saccharomyces cerevisiae* (reviewed in la Grange et al., 2010; van Zyl et al., 2007), *Hansenula polymorpha* (Voronovsky et al., 2009), *Kluyveromyces marxianus* (Hong et al., 2007; Yanase et al., 2010a) and *Scheffersomyces stipitis* (Morosoli et al., 1993).

Regardless of the process used for biomass hydrolysis, CBP-enabling microorganisms encounter a variety of toxic compounds (such as weak organic acids, furan derivatives, and phenolics) produced during biomass pretreatment that can inhibit microbial growth, metabolism and ethanol yield, particularly in the presence of ethanol (Almeida et al., 2007; Klinke et al., 2004; van Maris et al.,

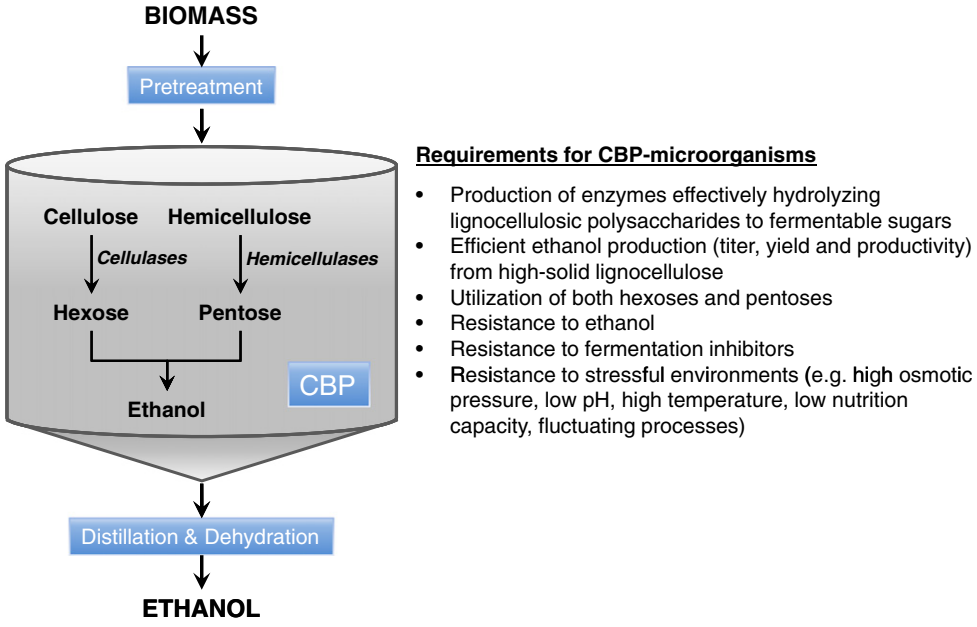

Fig. 1. Scheme for consolidated bioprocessing (CBP). The key to CBP is the engineering of a microorganism that can efficiently hydrolyze lignocellulosic polysaccharides to fermentable sugars and efficiently ferment this mixed-sugar hydrolysate to produce ethanol. Highly concentrated substrates required for an economically feasible process requires the microorganism to be robust with regard to inhibitors and osmotic tolerance. Furthermore, tolerance to ethanol, low pH, high temperature, low nutrition capacity and fluctuating processes is also desirable for a CBP microorganism.

2006). Consequently, methods for detoxifying lignocellulose hydrolysates are being developed (Cantarella et al., 2004; Klinke et al., 2004; Zhang et al., 2010), although large-scale detoxification is technically complex and contributes to the cost of the fermentation process. Alternatively, development of inhibitor-tolerant microorganisms has been carried out through the engineering of microorganisms.

Yeast, particularly *S. cerevisiae*, is usually the first choice for industrial ethanol production, because it has many advantages as an ethanol producer, such as faster sugar consumption, higher ethanol yield from glucose, and higher resistance to ethanol and other compounds present in lignocellulosic hydrolysates than bacteria (Lau et al., 2010; Olsson and Hahn-Hägerdal, 1993). Improved yeast tolerance to lignocellulosic hydrolysate has been achieved by overexpressing homologous and heterologous genes encoding traits responsible for inhibitor tolerance in *S. cerevisiae* strains (Larsson et al., 2001a,b; Sanda et al., 2011). In addition, adaptation and evolutionary engineering have led to obtaining inhibitor-tolerant strains in harsh environments which are typical in certain industrial fermentation conditions (Liu, 2006; Liu et al., 2009; Sauer, 2001). The yeast *S. cerevisiae* is also attractive because of its ease of genetic manipulation and is generally regarded as safe status due to its long association with the food and beverage industries. Thus, heterologous production of cellulases has been pursued with yeast hosts (Elkins et al., 2010; Lynd et al., 2002).

This review will focus on yeast strain development pursuant to the development of CBP. In particular, recent advances in heterologous expression of cellulolytic enzymes for direct conversion of cellulosic materials to ethanol are reviewed, and the improvement of yeast robustness by conferring tolerance to fermentation inhibitors is emphasized.

2. Bioconversion of lignocellulosic materials to ethanol

2.1. Hydrolysis of lignocellulosic materials

Lignocellulosic materials are composed mainly of cellulose, hemicellulose and lignin. Cellulose, a homopolymer of β-1,4-linked glucose units, is the major component of lignocelluloses and constitutes nearly 40 to 60% of its total dry weight (Gnansounou, 2008). The linear chains of cellulose are highly crystalline microfibrils. Hemicellulose, a substituted polysaccharide with a xylan backbone consisting of β-1,4-linked xylose monomers to which substituents and saccharides, such as glucose, galactose and arabinose, are attached, comprises 20 to 35% of lignocellulosic biomass (Saha, 2003). The hemicellulose molecules are linked to the cellulose microfibrils by hydrogen bonds. Lignin is a phenolic compound formed by polymerization of three types of monomers (i.e., *p*-coumaryl, coniferyl, and synapyl alcohol), which is present as an amorphous mass surrounding the cellulose and hemicellulose fibers (Grabber, 2005).

To hydrolyze cellulose into soluble sugars that can be metabolized by microbes, multiple enzymatic activities are required. These activities include endoglucanase (EG), exoglucanases including cellobiohydrolase (CBH) and cellodextrinase, and β-glucosidase (BGL) (Zhang et al., 2006). EG acts randomly against amorphous regions of cellulose chains to produce reducing and non-reducing ends for CBH, which produces cellobiose from reducing or non-reducing ends of crystalline cellulose. Cellulose chains are thus efficiently degraded to soluble cellobiose and cellooligosaccharides by the endo–exo synergism of EG and CBH (Medve et al., 1998; Teeri, 1997; Woodward, 1991). In the last step of enzymatic cellulose degradation, cellooligosaccharides are hydrolyzed to glucose by BGL. Exo–exo synergism between two cellobiohydrolases has also been reported (Medve et al., 1994). The filamentous fungus *T. reesei* is a well-known cellulolytic organism that secretes a cocktail of hydrolytic enzymes that degrade plant biomass, including at least two cellobiohydrolases (CBH1-2), five endoglucanases (EG1-5), as well as BGL and hemicellulases (Vinzant et al., 2001; Zhang and Lynd, 2004). The various cellulases synergistically act on the substrates, and the ratios of each cellulase are appropriately balanced by the regulation of cellulase gene expression in *T. reesei* to achieve the maximum hydrolysis in response to the environment (Sticker et al., 2008).

Apart from *Trichoderma* spp., the anaerobic bacteria *C. thermocellum* and *Clostridium cellulovorans* produce an elaborate enzyme complex known as a cellulosome, consisting of cellulase and hemicellulase organized on the cell surface (Doi and Tamaru, 2001; Shoham et al., 1999). The cellulosomal architecture involves multiple catalytic components assembled on a scaffoldin subunit through strong noncovalent protein–protein interactions between the cohesion modules on scaffoldin and the dockerin module contained in the individual cellulosomal enzymes (Himmel et al., 2010). This highly ordered structure of multiple enzymes in close proximity to the substrate results in a high level of enzyme–substrate–microbe synergy (Fierobe et al., 2005; Lu et al., 2006). Besides these species, the mesophilic cellulolytic *Clostridium cellulolyticum* has been widely exploited for its cellulosome (Desvaux, 2005). Cellulosomal organisms display a wide variety of catalytic subunits on the cell surface via scaffoldin. The catalytic diversity among subunits allows the organisms to modulate cellulosome activity according to the target substrate, lignocellulosic materials, by changing the composition of the catalytic subunits (Raman et al., 2009).

2.2. Secretion and cell surface display for development of CBP yeast

2.2.1. Secretion of cellulases for ethanol production from cellulosic materials

Heterologous expression and secretion of cellulases from either fungi or bacteria has been pursued during the last two decades. According to the review of van Zyl et al. (2007), most of the cellulases that have been successfully produced by *S. cerevisiae* were of fungal origin, although a number of bacterial cellulases have been expressed. The specific activity of recombinant CBH and BGL from *Aspergillus aculeatus* was comparable to that of the native enzyme (Takada et al., 1998). For the construction of a fully cellulolytic, fermentative system, multiple components of a cellulolytic system have been expressed by recombinant *S. cerevisiae* strains. Expression of the *Saccharomycopsis fibuligera* β-glucosidase-encoding gene enabled a recombinant *S. cerevisiae* strain to grow on cellobiose at approximately the same rate as on glucose in anaerobic conditions (van Rooyen et al., 2005). van Rensburg et al. (1998) constructed a yeast strain capable of hydrolyzing numerous cellulosic substrates and growing on cellobiose. den Haan et al. (2007b) reported coexpression of endoglucanase from *T. reesei* and β-glucosidase from *S. fibuligera* in a recombinant *S. cerevisiae* strain, which enabled its growth on a pure cellulose, phosphoric acid swollen cellulose (PASC), with direct production of ethanol. Anaerobic growth up to 0.27 g dry cell weight/l was observed on medium containing 10 g/l PASC as the sole carbon source with concomitant ethanol production up to 1.0 g/l (Table 1). Jeon et al. (2009) expressed a bacterial cellulose binding domain-containing endoglucanase from *C. cellulovorans* and β-glucosidase from *S. fibuligera* in a recombinant *S. cerevisiae* strain. The resultant yeast strain efficiently hydrolyzed cellulosic materials such as carboxymethylcellulose (CMC) and β-glucan to smaller fragments for efficient fermentation to ethanol through the cellulose binding domain's affinity to the substrate. Recently, a high-affinity cellodextrin transport system was isolated from the cellulolytic fungus, *Neurospora crassa* (Galazka et al., 2010). A recombinant *S. cerevisiae* strain expressing the cellodextrin transporter together with intracellular β-glucosidase grew on cellobiose and cellodextrin longer than cellobiose.

2.2.2. Cell surface display for ethanol production from cellulosic materials

Many attempts have been made to engineer *S. cerevisiae* strains to secret cellulases for the hydrolysis of cellulose (van Zyl et al., 2007). An alternative is the cell surface engineering to display the cellulolytic enzymes on the yeast cell surface. The use of the glycosylphosphatidylinositol anchoring system of the yeast cell surface has enabled display of various kinds of functional proteins on the cell surface without

Table 1
Ethanol production from cellulosic and hemicellulosic material using recombinant yeast strains expressing heterologous cellulases and hemicellulases.

Strain	Enzyme expressed	Enzyme-expression system	Carbon source	Ethanol production	Reference
S. cerevisiae	*T. reesei* EGII, *A. aculeatus* BGL1	Cell surface display	45 g/l barley β-glucan	16.5 g/l	Fujita et al. (2002)
S. cerevisiae	*T. reesei* XYNII, *A. oryzae* XylA/*S. stipitis* XR and XDH, *S. cerevisiae* XK	Cell surface display/ Intracellular expression	100 g/l birchwood xylan	7.1 g/l	Katahira et al. (2004)
S. cerevisiae	*T. reesei* EGII and CBHII, *A. aculeatus* BGL1	Cell surface display	10 g/l PASC	2.9 g/l	Fujita et al. (2004)
S. cerevisiae	*A. oryzae* EG and BGL	Cell surface display	20 g/l barley β-glucan	7.94 g/l	Kotaka et al. (2008)
S. cerevisiae	*T. reesei* EGI, *S. fibuligera* BGL1	Secretion	10 g/l PASC	1.0 g/l	den Haan et al. (2007b)
S. cerevisiae	*C. cellulovorans* EngD, *S. fibuligera* Bgl1	Secretion	20 g/l barley β-glucan, 20 g/l CMC	9.15 g/l, 11.03 g/l	Jeon et al. (2009)
S. cerevisiae/*E. coli*	Miniscaffoldin/Dockerin-tagged cellulases	Cell surface display/ Secretion	10 g/l PASC	3.5 g/l	Tsai et al. (2009)
H. polymorpha	*T. reesei* XYNII, *A. niger* xlnD	Secretion	90 g/l birchwood xylan	0.35 g/l	Voronovsky et al. (2009)
S. cerevisiae	Minicellulosome	Cell surface display	10 g/l PASC	1.87 g/l	Tsai et al. (2010)
S. cerevisiae	Minicellulosome	Cell surface display	10 g/l PASC	1.8 g/l	Wen et al. (2010)
K. marxianus	*T. reesei* EGII, *A. aculeatus* BGL1	Cell surface display	10 g/l barley β-glucan	4.24 g/l	Yanase et al. (2010a)
S. cerevisiae	*R. oryzae* GLAA, *S. bovis* AmyA, *T. reesei* EGII and CBHII, *A. aculeatus* BGL1	Cell surface display	10.2 g/l barley β-glucan, 8.44 g/l acid-treated Avicel[a], 50 g/l pretreated cassava pulp[b]	5.3 g/l, 1.04 g/l, 10 g/l	Apiwatanapiwat et al. (2011)
S. cerevisiae	*T. reesei* XYNII, *A. oryzae* XylA, *A. aculeatus* BGL1/*S. stipitis* XR and XDH, *S. cerevisiae* XK	Cell surface display/ Intracellular expression	rice straw hydrolysate[c]	8.2 g/l	Sakamoto et al. (2011)
S. cerevisiae	*T. reesei* EGII and CBHII, *A. aculeatus* BGL	Cell surface display	20 g/l PASC, 100 g/l pretreated rice straw[d]	7.6 g/l, 7.5 g/l	Yamada et al. (2011)

[a] Acid-treated Avicel was obtained by pretreatment with 1% H_2SO_4 at 150 °C for 2 h.
[b] Pretreated cassava pulp, containing app. 60% starch and app. 30% cellulose fiber, was prepared by hot-water treatment at 150 °C for 2 h.
[c] Rice straw hydrolysate, containing 37 g/l sugar component, was obtained as a water soluble fraction after liquid hot water treatment.
[d] Pretreated rice straw, containing 44.8% (w/w) glucan, was obtained as a water insoluble fraction after liquid hot water treatment.

the loss of their activity through genetic engineering (Kondo and Ueda, 2004). By displaying cellulolytic enzymes on the cell surface, efficient whole cell biocatalysts for simultaneous saccharification and fermentation have been constructed (Fujita et al., 2002, 2004; Kotaka et al., 2008; Yamada et al., 2011; Yanase et al., 2010b). The cell surface engineering system has an advantage where the cellulolytic enzymes are genetically self-immobilized on the yeast cell surface so that the activities of the enzymes are retained as long as the yeast continuous growing, while it is not easy to maintain the activities for a long reaction period in the conventional direct fermentation system in which the enzymes are secreted into the medium (Tanaka and Ueda, 2000). Another advantage of this system is easy separation of the biocatalyst from the product. Reutilization of the yeast cells enabled reuse of the enzymes displayed on their cell surface without reproduction of the yeast cells, which would reduce the cost of yeast propagation as well as enzyme addition (Kondo et al., 2002).

As a first step, Fujita et al. (2002) attempted to convert a cellulosic β-glucan from barley into ethanol by constructing cellulose degrading yeast cells that co-display *A. aculeatus* β-glucosidase 1 (BGL1) and *T. reesei* endoglucanase II (EGII) on the cell surface. Barley β-glucan is a linear, soluble polysaccharide composed of an average of 1200 glucose residues joined by approximately 70% β-1,4-glycosidic linkages and approximately 30% β-1,3-glycosidic linkages. The recombinant yeast showed growth in synthetic medium containing β-glucan as the sole carbon source and was able to directly ferment 45 g/l β-glucan to produce 16.5 g/l ethanol within 50 h. The yield was 0.48 g ethanol produced per gram carbohydrate utilized, which corresponds to 93.3% of the theoretical yield (Table 1). Subsequently, Fujita et al. (2004) reported simultaneous saccharification and fermentation of PASC to ethanol by the use of a recombinant yeast strain co-displaying three cellulolytic enzymes, *T. reesei* EGII and cellobiohydrolase II (CBHII) and *A. aculeatus* BGL1, on the cell surface. The yeast strain produced ethanol from PASC as the sole carbon source. The yield of ethanol was 0.45 g/g, which corresponds to 88.5% of the theoretical yield. By displaying three cellulolytic enzymes on the yeast cell surface, the cellobiose produced by the reaction of EGII and CBHII was further converted to glucose by BGL1 and the glucose taken up by the cell (Fig. 2). The study indicated that CBHII plays a very important role in cellulose degradation and that synergism between EGII and CBII is successfully induced on the yeast cell surface. Although surface-displayed CBHII had only a little activity with respect to PASC, the yeast strain codisplaying EGII and CBHII showed significantly higher activity than the yeast displaying only EGII and produced cellobiose as the main product (Fujita et al., 2004). Whole cell biocatalysts to degrade cellulose have several advantages: conversion of cellobiose and glucose, which inhibit cellulase and β-glucosidase activities; lower sterilization requirements, as glucose is immediately taken up by cells and ethanol is produced; and a requirement for a single reactor.

Although efficient degradation of lignocellulosic biomass requires an appropriate expression ratio of cellulolytic enzymes such as EG, CBH and BGL in recombinant *S. cerevisiae*, it is difficult to simultaneously control many different enzymes. Thus, Yamada et al. (2010) developed a method to optimize the cellulase expression ratio in yeast with a cocktail δ-integration system through an evolutionary engineering approach, which reveals the importance of EG expression for efficient PASC degradation. A diploid recombinant yeast strain optimized for expression of cellulolytic enzymes produced ethanol from agricultural waste biomass without the addition of exogenous enzymes (Yamada et al., 2011).

During the past couple of years, in a quest to extend cellulolytic capability, the minicellulosome has been displayed on the *S. cerevisiae* cell surface (Ito et al., 2009; Lilly et al., 2009; Tsai et al., 2009, 2010; Wen et al., 2010). Ito et al. (2009) constructed a cell surface display system by assembling (i) the Z domain of protein A and the Fc domain of human immunoglobulin G and (ii) the cohesin and dockerin domain from *C. cellulovorans* to construct chimeric scaffoldin at both the Z and cohesion domains. Heterologous *T. reesei* EGII and *A. aculeatus* BGL1, respectively fused with the Fc and dockerin domains, were expressed, secreted and assembled into functional complexes on the surface of the recombinant yeast cells via interactions with complementary domains on scaffoldin, yielding yeast strains capable of

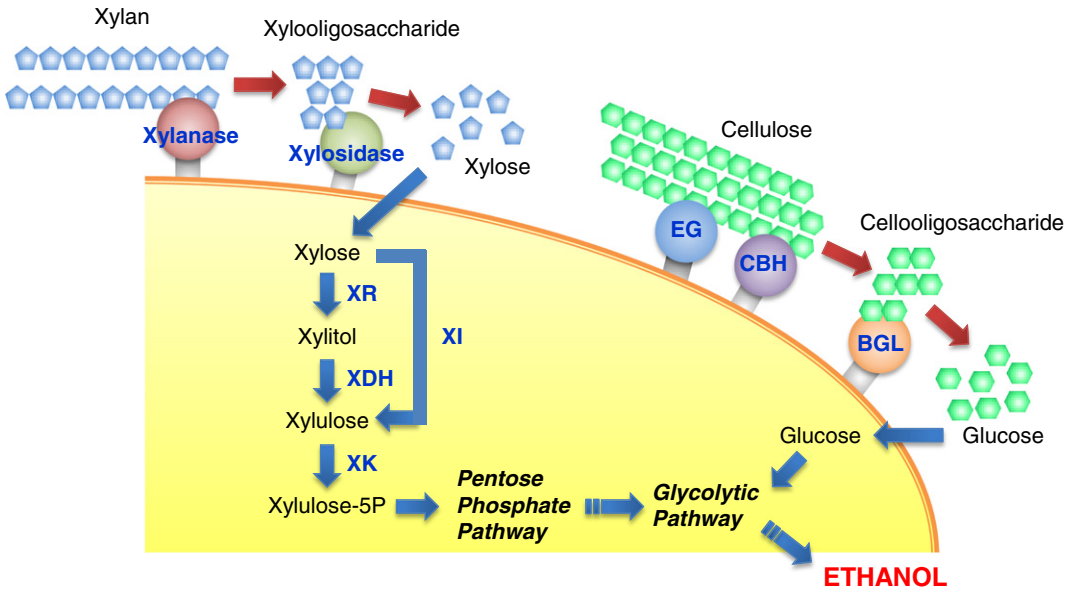

Fig. 2. Ethanol production pathway from cellulose and xylan using a recombinant *S. cerevisiae* strain displaying cellulolytic and hemicellulolytic enzymes such as endoglucanase (EG), cellobiohydrolase (CBH), β-glucosidase (BGL), xylanase and xylosidase on the cell surface and expressing xylose assimilating enzymes such as xylose reductase (XR), xylitol dehydrogenase (XDH) and xylulokinase (XK) in the cell. Xylulose-5P, xylulose-5-phosphate.

hydrolyzing β-glucan. Wen et al. (2010) reported engineering of *S. cerevisiae* strains displaying a trifunctional minicellulosome consisting of (i) a mini scaffoldin containing a cellulose binding domain and three cohesin modules, which was anchored to the cell surface and (ii) three types of cellulases, EGII, CBHII and BGL1, each bearing a C-terminal dockerin. Surface display of the trifunctional minicellulosomes gave yeast cells the ability to simultaneously break down and ferment PASC to ethanol with a titer of 1.8 g/l. The close proximity and ordering of EGII and CBHII on the miniscaffoldin enhanced the hydrolysis of PASC. Tsai et al. (2010) recently engineered yeast strains capable of either displaying a trifunctional scaffoldin carrying three divergent cohesin domains from *C. thermocellum*, *C. cellulolyticum* and *Ruminococcus flavefaciens*, or secreting one of the three corresponding dockerin-tagged cellulases (EG from *C. thermocellum*, exoglucanase from *C. cellulolyticum*, or BGL from *R. flavefaciens*). By the use of a yeast consortium composed of one strain displaying the mini scaffoldin and three strains secreting dockerin-tagged cellulases, the secreted cellulases were docked onto the displayed mini scaffoldin in a highly organized manner. Furthermore, by adjusting the ratio of different populations in the consortium, cellulose hydrolysis and ethanol production was successfully fine-tuned.

Also, cell surface engineering was applied to a thermotolerant strain of the yeast *K. marxianus* for the display of cellulolytic enzymes on the cell surface (Yanase et al., 2010a). One of the major drawbacks in CBP is the optimum temperature required for saccharification and fermentation. Saccharification with cellulolytic enzymes is best done around 50 °C, while most fermenting microbes have an optimum temperature for ethanol fermentation between 28 °C and 37 °C. Because the pretreatment of lignocellulose is at high temperature, the saccharification and fermentation temperatures should match those used in the previous process of CBP. Yanase et al. (2010a) reported that the recombinant *K. marxianus* strain codisplaying EG and BGL on the cell surface grew well at as high as 48 °C, at which it was able to produce ethanol from the cellulosic material β-glucan with a yield of 0.47 g ethanol per gram of consumed carbohydrate. This study supports the development of CBP yeast for effective bioethanol production.

The development of recombinant yeast strains displaying cellulases on the cell surface represents significant progress towards realization of CBP. High cell density suspensions of the recombinant strains fermented β-glucan, amorphous cellulose and pretreated cellulosic materials to produce ethanol (Table 1). Ideally, a large cell mass should be obtained by high-density cell culture under aerobic condition and cells harvested by sedimentation used for the ethanol fermentation. However, in the case of recombinant yeast, in which cellulases are expressed by secretion, this approach is not suitable because cells harvested should produce cellulases before ethanol fermentation. On the other hand, a higher ethanol production rate was achieved using yeast cells with surface cellulases, which were grown under aerobic condition.

It has been reported that CBHs are the key components for fungal cellulase systems (van Zyl et al., 2007). Wood (1992) noted that CBHs make up 80% of the total mass for the *T. reesei* system, and CBH1 plays a particularly important role, making up 60% of the total mass. However, den Haan et al. (2007a) reported that heterologous expression of fungal CBHs in *S. cerevisiae* significantly reduce their activity, presumably as a result of hyperglycosylation, but extracellular titers of CBHs were insufficient to allow direct conversion of cellulose to ethanol. In order to achieve efficient cellulose hydrolysis, increased expression of functional CBH should be a target for further research.

2.2.3. Direct ethanol production from hemicellulosic materials

The production of fuel ethanol from hemicellulosic hydrolysates is foreseen to be essential for the economic success of lignocellulosic ethanol (Gírio et al., 2010). Moreover, in the CBP strategy, cellulosic and hemicellulosic materials should be fermented simultaneously. Lignocellulosic material is often decomposed by acid treatment to release not only glucose but also various monosaccharides, such as xylose, mannose, galactose, and arabinose, and oligosaccharides in the hydrolysate. Therefore, microorganisms that are able to ferment these sugars are required for the industrial production of ethanol. However, a major disadvantage associated with using *S. cerevisiae* is its inability to utilize xylose, the most common pentose sugar in the hemicellulosic hydrolysate. Thus, most efforts in engineering of *S. cerevisiae* for xylose fermentation have focused on manipulation of the initial xylose metabolic pathway (reviewed in Hahn-Hägerdal et al., 2007; Nevoigt, 2008; van Vleet and Jeffries, 2009). Anaerobic xylose fermentation by *S. cerevisiae* was first performed by heterologous expression of xylose reductase (XR) and xylitol dehydrogenase (XDH) from *S. stipitis* together with overexpression of the

endogenous xylulokinase (XK) (Fig. 2). As another challenge in the fermentation of xylose, a xylose isomerase (XI) gene derived from the anaerobic fungi, *Piromyces* and *Orpinomyces*, has also been introduced into *S. cerevisiae* (Fig. 2). A characteristic difference between the XR/XDH strategy and the XI strategy is the $NADP^+$/NADH imbalance created between XR and XDH, which can result in the production of xylitol, thereby reducing ethanol yield (Karhumaa et al., 2007). However, the lack of hemicellulolytic capability of *S. cerevisiae* requires heterologous expression of hemicellulases for the direct conversion of hemicellulose to ethanol.

β-1,4-xylan, the most abundant component of hemicellulose, is a complex polysaccharide consisting of a backbone of β-1,4-linked xylopyranoside partially substituted with acetyl, glucuronosyl, and arabinosyl side chains. Xylan is hydrolyzed to xylooligosaccharides by endoxylanase, after which β-xylosidase hydrolyzes xylooligosaccharides to xylose (Biely, 1985; Kulkarni et al., 1999). A number of bacterial and fungal species are able to utilize xylan as a carbon source (Jeffries, 1983). *Trichoderma* spp. and *Aspergillus* spp. secrete large amounts of xylan-degrading enzymes (De Vries et al., 2000; Herrmann et al., 1997). Specifically, *T. reesei* secretes the two major inducible endoxylanases xylanase I (XYNI) and xylanase II (XYNII). XYNII represents more than 50% of the total xylanolytic activity of *T. reesei* cultivated on xylan (Törrönen et al., 1994). The industrial koji mold *Aspergillus oryzae* also produced and secreted efficiently xylan degrading enzymes such as β-xylosidase (Kitamoto et al., 1999).

Many researchers have studied the production of xylanolytic enzymes in the yeasts *S. cerevisiae* and *S. stipitis*. In particular, *S. cerevisiae* has been used as a host organism for the heterologous production of bacterial or fungal xylanases (la Grange et al., 1996; Li and Lijungdahl, 1996; Moreau et al., 1992; Pérez-Gonzalez et al., 1996) and β-xylosidase (la Grange et al., 1997; Margolles-Clark et al., 1996); attempts have been made to coproduce xylanase and β-xylosidase in this yeast as a means of converting xylan to cell mass or ethanol through simultaneous saccharification and fermentation (la Grange et al., 2001). Katahira et al. (2004) reported the display of both *T. reesei* XYNII and *A. oryzae* β-xylosidase on the cell surface of *S. cerevisiae*, which made it possible to hydrolyze birchwood xylan to xylose. They also introduced XR, XDH and XK into the xylan-degrading yeast strain, which enabled simultaneous saccharification and fermentation of xylan (Fig. 2). After 62 h of fermentation, 7.1 g/l ethanol was directly produced from birchwood xylan, and the yield was 0.30 g ethanol per gram carbohydrate (Table 1). The report demonstrated that the combination of a cell surface display system and additional metabolic functions is effective in the construction of yeast cells capable of multistep bioconversion.

Xylanolytic enzymes were also expressed in *S. stipitis* with the ability to ferment xylose (den Haan and van Zyl, 2001, 2003; Morosoli et al., 1993). The direct production of ethanol from xylan was performed using a recombinant *S. stipitis* strain secreting *Cryptococcus albidus* xylanase, although the ethanol productivity and yield were not reported (Morosoli et al., 1993). Recently, Voronovsky et al. (2009) engineered a thermotolerant yeast, *H. polymorpha*, coexpressing endoxylanase from *T. reesei* and β-xylosidase from *Aspergillus niger* by integrating these genes into the *H. polymorpha* genome with the promoter of the *H. polymorpha* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The resulting transformants were capable of growth and ethanol production on a minimal medium supplemented with birchwood xylan as the sole carbon source at 48 °C.

Although the fermentation of a xylose-cellobiose mixture is simultaneously performed by the thermotolerant methylotrophic yeast *H. polymorpha* (Ryabova et al., 2003), the ethanol yield and productivity are insufficient. Thus, Katahira et al. (2006) constructed a recombinant *S. cerevisiae* strain with xylose fermenting ability by introducing genes of *S. stipitis* XR and XDH and *S. cerevisiae* XK for intracellular expression, as well as cellooligosaccharide degrading ability by introducing a fusion gene of *A. aculeatus* BGL1 and the 3′ half of α-agglutinin for cell-surface display. Using the recombinant strain, ethanol was produced from the lignocellulosic hydrolysate obtained by concentrated sulfuric acid pretreatment of wood chips.

Recently, Sakamoto et al. (2011) successfully produced ethanol from a rice straw hydrolysate obtained by liquid hot water treatment. The treatment solubilizes the hemicellulosic component of rice straw to yield liquid-soluble fractions containing not only cellooligosaccharide but also xylan and xyloglucan. Accordingly, recombinant XR/XDH/XK-based *S. cerevisiae* strains co-displaying *A. aculeatus* BGL, *A. oryzae* β-xylosidase and *T. reesei* endoxylanase on the cell surface was developed, which directly produced ethanol from the hemicellulosic materials without a requirement for addition of sugar-hydrolyzing enzymes or detoxification of the hydrolysate. The ethanol yield (grams of ethanol per gram of total sugar in rice straw hydrolysate consumed) was 0.41 g/g, which corresponded to 82% of the theoretical yield.

3. Development of yeast strains resistant to fermentation inhibitors

3.1. Fermentation inhibitors

The task of hydrolyzing lignocellulose to fermentable sugars is still technically problematic because the digestibility of cellulose is hindered by the structure and composition of lignocellulosic biomass (Himmel et al., 2007). Owing to this structural recalcitrance, a chemical and physicochemical pretreatment (such as acid pretreatment, lime treatment, steam explosion, liquid hot water treatment, or ammonia fiber explosion) is an essential step to obtain potentially fermentable sugars in the hydrolysis step (Alvira et al., 2010). In the context of biological conversion of lignocellulosic materials to ethanol, the pretreatment is required to separate lignin by breaking down its structure and to disrupt the crystalline structure of cellulose to facilitate the access of enzymes to the cellulose substrates during the hydrolysis step (Alvira et al., 2010; da Costa Sousa et al., 2009; Hendriks and Zeeman, 2009).

However, the harsh conditions used in the pretreatment of the raw material release fermentation inhibitors including weak organic acids (particularly acetic and formic acids), furan derivatives, and phenolic compounds (reviewed in Almeida et al., 2007; Liu and Blaschek, 2009; Palmqvist and Hahn-Hägerdal, 2000; van Maris et al., 2006). Acetic acid released during solubilization and hydrolysis of biomass is generally found at a high concentration in the lignocellulosic hydrolysate (Klinke et al., 2003; Larsson et al., 1999; Thomsen et al., 2009; Tomás-Pejó et al., 2010), with the levels varying depending on the biomass type and pretreatment method. Formic acid is typically present at lower concentrations than acetic acid, but is more toxic to *S. cerevisiae* (Hasunuma et al., 2011a; Martin et al., 2007). Furan derivatives such as 2-furaldehyde (furfural) and 5-hydroxymethyl-2-furaldehyde (5-hydroxymethylfurfural; 5-HMF) are formed by dehydration of pentoses and hexoses, respectively. A wide range of phenolic compounds such as vanillin, syringaldehyde, 4-hydroxybenzaldehyde and ferulic acid are generated due to lignin breakdown.

Although the mechanism of inhibition by weak acids is not easily elucidated, the inhibitory effect of weak acids has been ascribed to uncoupling and intracellular anion accumulation (Russell, 1992) (Fig. 3). The undissociated form of weak acids diffuses from the fermentation medium across the plasma membrane and dissociates due to the higher intracellular pH, thus decreasing the cytosolic pH (Guldfeldt and Arneborg, 1998). The intracellular acidification inhibits cell metabolic activity (Pampulha and Loureiro-Dias, 1990) and contributes to the dissipation of plasma membrane electrochemical gradient (Mira et al., 2010b). In glycolysis, enolase was the enzyme most severely affected by acetic acid at pH6.6 (Pampulha and Loureiro-Dias, 1990). The decrease in intracellular pH can be compensated by the proton-translocating plasma membrane ATPase, Pma1, which pumps protons out of the cell at the expense of ATP hydrolysis

(Carmelo et al., 1997) (Fig. 3). As a consequence, less ATP is available for biomass formation. Also, the depletion of ATP should affect ATP-dependent enzymes including hexokinase and phosphofructokinase in glycolysis (Pampulha and Loureiro-Dias, 1990). Intracellular acidification leads to decreased DNA and RNA synthesis rate (Madshus, 1988). Besides affecting internal pH homeostasis, weak acids also have an impact on the lipid organization and function of membrane embedded protein (Fernandes et al., 2005; Piper et al., 1998). Moreover, extensive degradation of ribosomal RNA is observed in acetic acid-stressed cells as a result of activation of apoptotic mechanisms (Mroczek and Kufel, 2008).

Furan aldehydes such as furfural and 5-HMF prevent yeast cells from growing and producing ethanol (Navarro, 1994; Palmqvist et al., 1999; Sanchez and Bautista, 1988). Several experimental results may explain the inhibitory effects on ethanol fermentation. *In vitro* measurements showed that alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH), and pyruvate dehydrogenase (PDH) were directly inhibited by furfural and 5-HMF (Modig et al., 2002). When yeast cells were grown in the presence of furfural, inhibition of GAPDH was observed (Banerjee et al., 1981). The reduction of furfural and 5-HMF to 2-furanmethanol (FM) and furan-2,5-dimethanol (FDM) in yeast cells should affect intracellular redox balance by the NAD(P)H oxidation (Liu, 2011; Palmqvist et al., 1999). Also, furan aldehydes are implicated in a broad range of effects including cell wall damage, DNA breakdown, inhibition of protein and RNA synthesis (Khan and Hadi, 1994; Liu, 2011; Sanchez and Bautista, 1988). Recently, Allen et al. (2010) showed that furan aldehydes also induced the accumulation of reactive oxygen species (ROS) in cells to cause damages to mitochondria and vacuole membranes, the actin cytoskeleton and nuclear chromatin, when healthy and exponentially growing cells were transferred to furfural (Fig. 3).

Although the mechanism of metabolic inhibition by phenolic compounds derived from lignin have not yet been elucidated, largely due to the lack of accurate quantitative analysis, the phenolic compounds have been suggested to act on biological membranes, causing loss of integrity, thereby affecting their ability to serve as selective barriers and enzyme matrices (Heipieper et al., 1994; Larsson et al., 2000; Palmqvist and Hahn-Hägerdal, 2000) (Fig. 3).

To exploit lignocellulosic materials for fuel ethanol production, the fermenting yeast must utilize hexose and pentose sugars in the presence of these inhibitory compounds. In particular, xylose utilization by recombinant xylose fermenting recombinant *S. cerevisiae* strains was more severely affected by the addition of acetic acid than glucose utilization (Hasunuma et al., 2011a). Thus, in order to improve fermentation ability of the yeast strains, several strategies have been applied to overcome the effect of inhibitors. These approaches include the improvement of natural tolerance by controlling inhibitor concentrations during the fermentation (Martín et al., 2007), a mutagenesis and genome shuffling approach (Liu et al., 2009; Zheng et al., 2011), and the overexpression of genes encoding enzymes that confer resistance towards specific inhibitors, as described in the following sections.

3.2. Evolutionary engineering for the improvement of inhibitor tolerance

Evolutionary engineering strategies were proved to be useful to improve fermentation ability of constructed strains by mimicking natural selection under pressure or random mutagenesis (Sonderegger and

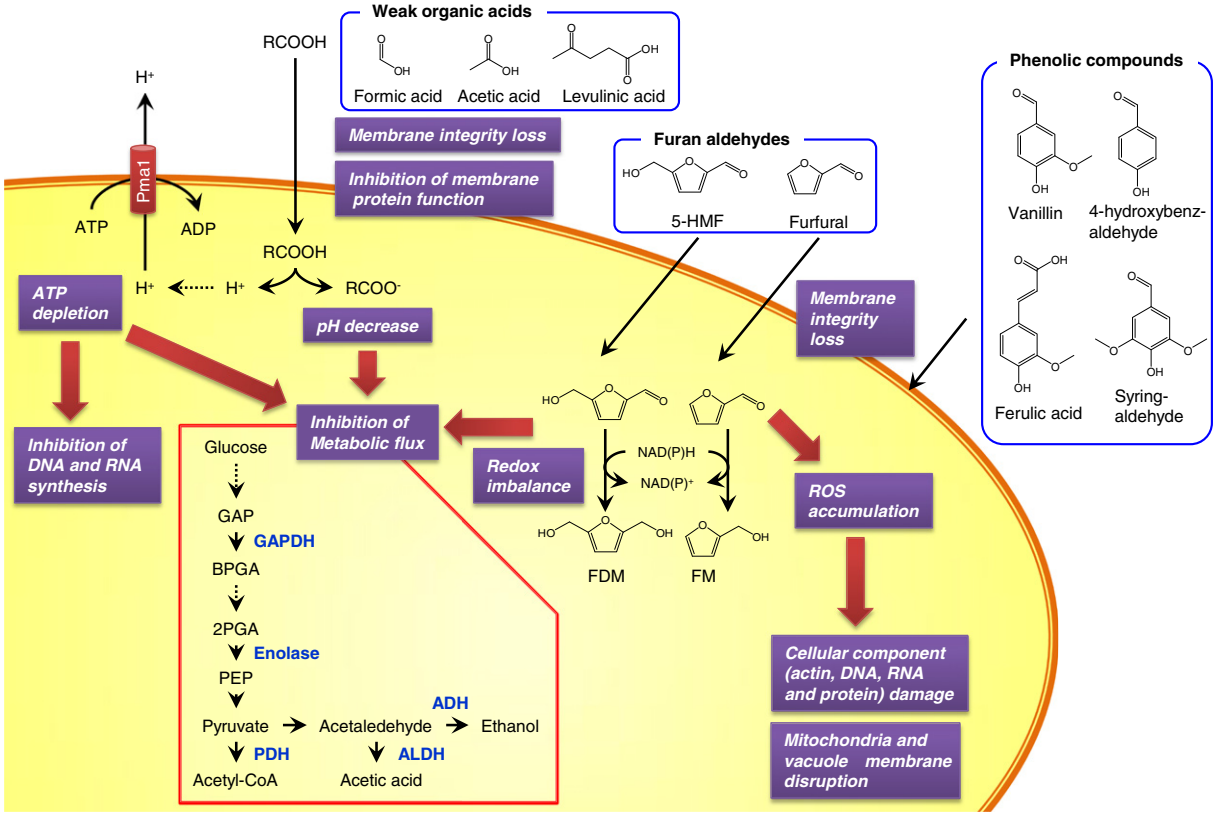

Fig. 3. Schematic representation of a presumed inhibition mechanism of weak acids, furan derivatives and phenolic compounds in *S. cerevisiae*. Undissociated form of weak acids diffuses across plasma membrane and dissociates in the cells, thus decreasing the cytosolic pH. H^+-ATPase, Pma1 activity contributes to the recovery of internal pH at the expense of ATP hydrolysis. The intracellular acidification and ATP depletion inhibit cell metabolic activity. Weak acids also have an impact on the membrane organization and function of membrane embedded protein. Furan aldehydes induce ROS accumulation in cells to cause damages to mitochondria and vacuole membranes as well as cellular components. Furan aldehydes prevent yeast cells from growing and producing ethanol. The reduction of furfural and 5-HMF to FM and FDM causes intracellular redox imbalance by NAD(P)H oxidation. Glycolytic enzymes, ADH, ALDH, enolase, GAPDH and PDH, are inhibited by weak acids and furan aldehydes. Phenolic compounds cause loss of plasma membrane integrity. BPGA, 1,3-*bis*phosphoglycerate; GAP, glyceraldehyde 3-phosphate; PEP, phospho*enol*pyruvate; 2PGA, 2-phosphoglycerate.

Sauer, 2003; Wahlbom et al., 2003). Evolutionary engineering experiments were successful in obtaining inhibitor tolerant strains, as well as to accelerate the co-utilization of glucose, xylose, and arabinose mixtures by a recombinant *S. cerevisiae* strain (Wisselink et al., 2009). Martín et al. (2007) reported that the adaptation of xylose-fermenting *S. cerevisiae* strains to sugarcane bagasse by cultivation over 353 h using a medium with an increasing concentration of fermentation inhibitors led to a significant improvement of ethanol production from bagasse hydrolysate. The adapted strains converted furfural and 5-HMF at a faster rate than the non-adapted strain. Tomás-Pejó et al. (2010) improved biomass fermentation ability of a recombinant xylose-fermenting *S. cerevisiae* through evolutionary engineering. The evolved strain, obtained by sequential cultivation in wheat straw hydrolysate with an increasing concentration of inhibitors, exhibited 20% higher final ethanol concentration and 65% higher xylose consumption than the parental strain in the fermentation of steam-pretreated wheat straw. In a previous report (Basso et al., 2008), several *S. cerevisiae* strains suitable for fermentation of sugarcane substrates were isolated by cell recycling under the stressful conditions imposed by industrial fermentation. The selection of yeast strains under selective pressure may improve their fermentation performance in terms of high ethanol yield, reduced glycerol and foam formation, and maintenance of high viability during recycling. Cell recycling may also have the advantage of allowing cells to become conditioned or adapted to the fermentation process (Slininger et al., 2011).

3.3. Genetic engineering for the improvement of inhibitor tolerance

3.3.1. In situ detoxification of fermentation inhibitors

In order to improve ethanol production in the presence of fermentation inhibitors, a genetic engineering approach using enzymes responsible for the inhibitor tolerance of *S. cerevisiae* strains was launched (Table 2). Overexpression of homologous or heterologous genes encoding these enzymes has been successfully applied to the detoxification of inhibitors such as furan derivatives and phenolic compounds (Almeida et al., 2009; Gorsich et al., 2006; Heer et al., 2009; Larsson et al., 2001a,b; Liu and Blaschek, 2009).

Several strains of *S. cerevisiae* showed tolerance to furans due to their ability to convert furfural and 5-HMF to less harmful compounds, respectively FM and FDM (Liu et al., 2004, 2008). The alcohol dehydrogenase encoded by the *ADH6* gene is one of the enzymes responsible for NADPH-dependent 5-HMF reduction in *S. cerevisiae* (Petersson et al., 2006). Also, Laadan et al. (2008) identified an ADH1 variant as an NADH-dependent enzyme capable of reducing both furfural and 5-HMF from the industrial *S. cerevisiae* yeast strain TMB3000, which was the most tolerant strain in a screening test using dilute acid hydrolysate, while the natural ADH1 enzyme from *S. cerevisiae* is not able to reduce 5-HMF. Accordingly, the overexpression of these ADH genes in yeast cells has aided ethanol production from glucose or a glucose/xylose mixture in the presence of furfural or 5-HMF (Almeida et al., 2008, 2009; Laadan et al., 2008; Petersson et al., 2006). Recently, Liu and Moon (2009) reported that the aldehyde reductase encoded by *ARI1* possessed NADPH-dependent reduction activities toward at least 14 aldehydes including common lignocellulose-derived inhibitors such as furfural, 5-HMF, vanillin, and cinnamic aldehyde. Cultures of the *S. cerevisiae* strain overexpressing *ARI1* demonstrated a better growth compared with a wild type strain without the gene insert in the presence of 20 mM furfural and 40 mM HMF (Liu and Moon, 2009). It was likely attributed to its enzyme reduction activities to convert the inhibitors into less toxic or non-toxic alcohols that allow cell growth. On the other hand, deletion of a single gene encoding enzymes possessing aldehyde reduction activities did not affect cell growth in the presence of the inhibitors, suggesting in situ detoxification of furfural and 5-HMF by furan-tolerant yeast strains involves multiple gene mediated NAD(P)H-dependent aldehyde reduction (Liu and Moon, 2009; Liu et al., 2008). Individual enzymes involved in reduction of furfural and 5-HMF had distinct cofactor preference. ADH7, ALD4, and GRE3 demonstrated clearly cofactor NADH preference while ADH6 showed NADPH preference on both substrates, furfural and 5-HMF. Therefore, Liu et al. (2008, 2009) indicated that either NADH and/or NADPH can be used for the aldehyde reduction.

To detoxify phenolic inhibitors, laccase from the white rot fungus *Trametes versicolor* was expressed in *S. cerevisiae*. The laccase-expressing strain had the ability to convert coniferyl aldehyde at a faster rate than a control strain not expressing laccase, which enabled faster growth and ethanol fermentation in the presence of 1.25 mM coniferyl aldehyde (Larsson et al., 2001a). Also, the overexpression of the *Pad1* gene, encoding phenylacrylic acid decarboxylase (Pad1p), improved ethanol productivity in the presence of ferulic acid and cinnamic acid (Larsson et al., 2001b). The Pad1p-overexpressing strain also showed 22–25%, 40–45%, and 24–29% faster glucose consumption rate, mannose consumption rate, and ethanol production rate, respectively, in a dilute acid hydrolysate of spruce under aerobic and oxygen-limited conditions. As described above, ARI1 from *S. cerevisiae* has aldehyde reduction activities toward lignin-derived phenolic compounds such as benzoic aldehyde, cinnamic aldehyde, and vanillin (Liu and Moon, 2009). Also, ADH7 possessing reduction activities to both furfural and 5-HMF showed reduction activities with cinnamic aldehyde (Liu et al., 2008). Overexpression of *ARI1* and *ADH7* in recombinant yeast strains is of interest for in situ detoxification of phenolic aldehydes present in lignocellulosic hydrolysates.

3.3.2. Improvement of inhibitor tolerance through metabolic engineering based on systems biology

The inherent strategy of yeast to counteract the negative impact of inhibitors, particularly in the case of furans and other aldehydes, is to reduce them to less toxic compounds, such as alcohol. On the other hand, little is known about the molecular and physiological basis of yeast tolerance to the major inhibitors. Accordingly, the mechanisms of tolerance and adaptation of *S. cerevisiae* towards lignocellulosic hydrolysate have been the subject of many studies, and would allow the improvement of inhibitor tolerance through metabolic engineering. In particular, systems biology approaches including disruptome screening, a genome-wide approach using DNA microarrays, proteomics, and metabolomics have been recently exploited to gain insight into the molecular and genetic traits involved in tolerance and adaptation to fermentation inhibitors (Gorsich et al., 2006; Hasunuma et al., 2011a,b; Heer et al., 2009; Lin et al., 2009; Ma and Liu, 2010; Mira et al., 2010a,b; Sundström et al., 2010).

To identify genes involved in furfural tolerance, Gorsich et al. (2006) screened an *S. cerevisiae* gene disruption library for mutants that exhibit growth deficiencies in the presence of furfural. Interestingly, several mutants defective in the pentose phosphate pathway (PPP) genes, *zwf1*, *gnd1*, *rpe1*, and *tkl1*, were found to be inefficient at reducing furfural to furfuryl alcohol. These data might reflect an overall decreased abundance of reducing equivalents or the role of NADPH in stress tolerance. Indeed, the overexpression of the *zwf1* gene, encoding glucose-6-phosphate dehydrogenase, in *S. cerevisiae* enabled growth at furfural concentrations that are normally toxic (Table 2). Recently, the functional basis of furfural tolerance was elucidated by comparing a furfural-adapted *S. cerevisiae* strain with increased viability under high concentrations of furfural to its parent by means of a metabolic flux and global transcript analysis (Heer et al., 2009). Although NADH-dependent oxidoreductases are the major defense system at lower concentrations of furfural, at furfural concentrations above 15 mM, the NADPH-dependent oxidoreductases become the major resistance mechanism with increased NADPH-generating flux through the PPP. Furthermore, Heer et al. (2009) demonstrated that high-level furfural resistance in the adapted strain was based on strong induction of *ADH7*, the

Table 2
Improved tolerance toward fermentation inhibitors through genetic engineering.

Genetic engineering strategy	Gene-product function	Major impact on inhibitor tolerance of *S. cerevisiae* strains	Reference
T. versicolor lcc2 and *S. cerevisiae* Sso2p overexpression[a]	Laccase and protein secretion	Increased growth and ethanol production from glucose in the presence of coniferyl aldehyde under oxygen-limited conditions by detoxifying coniferyl aldehyde	Larsson et al. (2001a)
Pad1 overexpression	Phenylacrylic acid decarboxylase	Increased growth and ethanol production from glucose in the presence of ferulic and cinnamic acid under oxygen-limited conditions	Larsson et al. (2001b)
ZWF1 overexpression	Pentose phosphate pathway	Increased growth in synthetic dextrose medium in the presence of furfural	Gorsich et al. (2006)
ADH6 overexpression	Aldehyde reduction	Increased NADPH-dependent HMF conversion under aerobic and anaerobic cultivation	Petersson et al. (2006)
		Increased growth and ethanol production in anaerobic glucose fermentation in the presence of HMF	Almeida et al. (2008)
		Increased growth in synthetic dextrose medium in the presence of HMF	Liu et al. (2008)
		Increased ethanol production from glucose-xylose mixture in the presence of HMF	Almeida et al. (2009)
Mutated *ADH1* overexpression	Aldehyde reduction	Increased growth and ethanol production in anaerobic glucose fermentation in the presence of HMF	Almeida et al. (2008)
		Increased biomass production and NADH-dependent HMF conversion	Laadan et al. (2008)
		Increased ethanol production from glucose-xylose mixture in the presence of HMF	Almeida et al. (2009)
ADH7 overexpression	Aldehyde reduction	Increased growth in synthetic dextrose medium in the presence of HMF	Liu et al. (2008)
ADH7 and *YKL071W* overexpression	Aldehyde reduction	Reduction of furfural-induced lag phase	Heer et al. (2009)
ARI1 overexpression	Aldehyde reduction	Increased growth in a minimal medium in the presence of furfural and HMF	Liu et al. (2009)
FPS1[b] disruption	Aquaglycoporin	Increased growth and ethanol production from glucose in the presence of acetic acid	Zhang et al. (2011)
TAL1 overexpression	Pentose phosphate pathway	Increased ethanol production from xylose in the presence of acetic and formic acid	Hasunuma et al. (2011a)
FDH1 overexpression	Formate dehydrogenase	Detoxification of formic acid during xylose fermentation	Hasunuma et al. (2011b)
TAL1 and *FDH1* overexpression	Pentose phosphate pathway, Formate dehydrogenase	Increased ethanol production in a repeated-batch fermentation of rice straw hydrolysate[c]	Sanda et al. (2011)

[a] The Sso2 protein is a membrane protein involved in the protein secretion machinery.
[b] The *FPS1* gene encodes an aquaglycoporin protein involved in glycerol uptake and efflux.
[c] Rice straw hydrolysate obtained by liquid hot water pretreatment and hemicellulose treatment contained sugars (11.99 g/l glucose, 9.66 g/l xylose, 2.58 g/l arabinose, 0.89 g/l galactose, 4.72 g/l fructose), 27.11 mM acetate, 20.06 mM formate, 7.77 mM furfural, 0.46 mM 5cvHMF, 0.56 mM vanillin and 0.37 mM syringaldehyde.

uncharacterized open reading frame *YKL071W*, and four additional (likely NADPH-dependent) oxidoreductases. By overexpressing the *ADH7* genes and the open reading frame *YKL071W*, furfural resistance was increased in the parent strain, which demonstrated these two enzymes are the key elements of furfural resistance.

In contrast to furans and phenolic compounds, metabolic engineering strategies that have addressed yeast tolerance to weak organic acids have been rare, due to the insufficient understanding of inhibitory actions of weak organic acids. Recently, the effect of acetic acid on xylose fermentation was analyzed by examining metabolite profiles in a recombinant xylose fermenting strain of *S. cerevisiae* (Hasunuma et al., 2011a). Metabolome analysis revealed that metabolites involved in the non-oxidative PPP accumulated significantly on addition of acetic acid, indicating that acetic acid may slow down the flux through the pathway. Subsequently, the overexpression of the *TAL1* gene encoding transaldolase, which is assumed to be one of the rate-limiting enzymes in the non-oxidative PPP, reduced the inhibition of the fermentation capacity by weak acids such as acetic and formic acids. This study demonstrated that metabolomics is a powerful tool to gain insight into the effects of environmental perturbation on microbial metabolism and to then develop rational strategies for conferring stress tolerance through genetic engineering.

Microarray analysis has also been applied to survey the transcriptional response of *S. cerevisiae* to stress, a strategy that has uncovered many unpredictable stress responses (Li and Yuan, 2010; Mira et al., 2010a). Microarray analysis of xylose fermenting *S. cerevisiae* under different formic acid concentrations revealed that the expression of 7 genes (*FDH1*, *FDH2*, *ALD5*, *HXK2*, *VCX1*, *GPD2*, and *HXT4*) was upregulated in response to increasing formic acid dosage (Hasunuma et al., 2011b). The list of upregulated genes suggested a response of *S. cerevisiae* to formic acid, and the major defense mechanism is likely to be upregulation of formate dehydrogenase (FDH) activity. In order to consume the NADH produced by formic acid breakdown, transcription of two NAD-dependent reductases, alcohol dehydrogenase (*ALD5*) and glycerol-3-phosphate dehydrogenase (*GPD2*), were induced in an attempt to maintain the redox balance. Genes in the initial steps for sugar assimilation, encoding a hexose transporter (*HXT4*) and hexokinase (*HXK2*), were also upregulated to produce more ATP. Quantification of formic acid in the medium indicated that the innate activity of FDH was too weak to detoxify formic acid. To reinforce the capability for formic acid breakdown, the *FDH1* gene was also overexpressed in the xylose-fermenting recombinant yeast strain. This modification allowed the yeast to rapidly decompose excess formic acid (Hasunuma et al., 2011b).

Based on knowledge obtained by biological profiling (Hasunuma et al., 2011a, 2011b), a recombinant *S. cerevisiae* strain that demonstrated higher ethanol production from xylose in the presence of both acetic and formic acid was developed by expressing both the *TAL1* and *FDH1* genes (Sanda et al., 2011). As an additional strategy for enhancing the resistance to weak acids, the haploid TAL/FDH-coexpressing xylose-fermenting strains were crossbred to obtain a homozygous diploid strain. The recombinant diploid strain produced ethanol from lignocellulosic hydrolysate containing a mixture of glucose, fructose and xylose as a carbon sources, as well as the fermentation inhibitors. Moreover, batch fermentation of the lignocellulosic hydrolysate was performed for five cycles without any loss of fermentation ability (Sanda et al., 2011).

4. Conclusions

Cost-effective production of ethanol from lignocellulosic materials remains a major challenge, primarily due to the high recalcitrance of these materials. Typically, high cost is associated with the large quantity of enzymes required for the complete hydrolysis of cellulose to ethanol. The development of cell surface engineering provides efficient yeast cell biocatalysts directly converting lignocellulosic materials to ethanol by displaying cellulolytic and hemicellulolytic enzymes on the cell surface. The close proximity of multiple enzymes on the cell surface enables synergistic hydrolysis of the lignocellulosic materials. Furthermore, reutilization of the yeast cells enables reuse of the enzymes displayed on their cell surface without reproduction of the yeast cells, which would reduce the cost of yeast propagation as well as enzyme addition. Thus, cell surface engineering is a promising technology for the realization of CBP.

For the efficient production of ethanol from lignocellulosic materials, the improvement of not only fermentation capacity but also tolerance to toxic compounds released by pretreatment of biomass is indispensable. However, limited knowledge is available about the molecular and physiological basis of yeast tolerance to the inhibitory compounds. Thus, evolutionary engineering is a useful strategy to obtain a robust strain, while an important challenge for metabolic engineering is identification of gene targets that have importance for improvement of cellular robustness. Tolerant yeast strains can be obtained by enhancing the genetic background. A systems biology approach using multi-omics analysis such as transcriptomics, proteomics and metabolomics, which present a dynamic view of gene expression, protein and metabolite biosynthesis in yeast stress responses, will facilitate metabolic engineering to optimize microorganisms for industrial ethanol production from lignocellulosic materials.

A combination of cell surface engineering and intracellular metabolic engineering is a very effective approach to develop cells with novel fermentation ability for industrial applications, which would be applicable to the development of functional consolidated bioprocessing. This technology will open up new applications of cell factories for the future of white biotechnology.

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References

Allen SA, Clark W, McCaffery JM, Cai Z, Lanctot A, Slininger PJ, et al. Furfural induces reactive oxygen species accumulation and cellular damage in *Saccharomyces cerevisiae*. Biotechnol Biofuel 2010;3:2.

Almeida JRM, Modig T, Petersson A, Hahn-Hägerdal B, Lidén G, Gorwa-Grauslund MF. Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. J Chem Technol Biotechnol 2007;82:340–9.

Almeida JR, Röder A, Modig T, Laadan B, Lidén G, Gorwa-Grauslund MF. NADH- vs NADPH-coupled reduction of 5-hydroxymethyl furfural (HMF) and its implications on product distribution in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 2008;78:939–45.

Almeida JRM, Bertilsson M, Hahn-Hägerdal B, Lidén G, Gorwa-Grauslund MF. Carbon fluxes of xylose-consuming *Saccharomyces cerevisiae* strains are affected differently by NADH and NADPH usage in HMF reduction. Appl Microbiol Biotechnol 2009;84:751–61.

Alvira P, Tomás-Pejó E, Ballesteros M, Negro MJ. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. Bioresour Technol 2010;101:4851–61.

Anasontzis GE, Zerva A, Stathopoulou PM, Haralampidis K, Diallinas G, Karagouni AD, et al. Homologous overexpression of xylanase in *Fusarium oxysporum* increases ethanol productivity during consolidated bioprocessing (CBP) of lignocellulosics. J Biotechnol 2011;152:16–23.

Apiwatanapiwat W, Murata Y, Kosugi A, Yamada R, Kondo A, Arai T, et al. Direct ethanol production from cassava pulp using a surface-engineered yeast strain co-displaying two amylases, two cellulases, and β-glucosidase. Appl Microbiol Biotechnol 2011;90:377–84.

Banerjee N, Bhatnagar R, Viswanathan L. Inhibition of glycolysis by furfural in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 1981;11:226–8.

Basso LC, de Amorim HV, de Oliveila AJ, Lopes ML. Yeast selection for fuel ethanol production in Brazil. FEMS Yeast Res 2008;8:1155–63.

Biely P. Microbial xylanolytic systems. Trends Biotechnol 1985;3:286–90.

Cantarella M, Cantarella L, Gallifuoco A, Spera A, Alfani F. Comparison of different detoxification methods for steam-exploded poplar wood as a substrate for the bioproduction of ethanol in SHF and SSF. Process Biochem 2004;39:1533–42.

Cardona CA, Sánchez OJ. Fuel ethanol production: process design trends and integration opportunities. Bioresour Technol 2007;98:2415–57.

Carmelo V, Santos H, Sá-Correia I. Effect of extracellular acidification on the activity of plasma membrane ATPase and on the cytosolic and vacuolar pH of *Saccharomyces cerevisiae*. Biochim Biophys Acta 1997;1325:63–70.

da Costa Sousa L, Chudawat SPS, Balan V, Dale BE. Cradle-to-grave assessment of existing lignocellulose pretreatment technologies. Curr Opin Biotechnol 2009;20:339–47.

De Vries RP, Kester HCM, Poulsen CH, Benen JAE, Visser J. Synergy between enzymes from *Aspergillus* involved in the degradation of plant cell wall polysaccharides. Carbohydr Res 2000;327:401–10.

Demain AL, Newcomb M, Wu JHD. Cellulase, Clostridia, and ethanol. Microbiol Mol Biol Rev 2005;69:124–54.

den Haan R, van Zyl WH. Differential expression of the *Trichoderma reesei* β-xylanase II (*xyn2*) gene in the xylose-fermenting yeast *Pichia stipitis*. Appl Microbiol Biotechnol 2001;57:521–7.

den Haan R, van Zyl WH. Enhanced xylan degradation and utilisation by *Pichia stipitis* overproducing fungal xylanolytic enzymes. Enzyme Microb Technol 2003;33:620–8.

den Haan R, McBride JE, la Grange DC, Lynd LR, van Zyl WH. Functional expression of cellobiohydrolyases in *Saccharomyces cerevisiae* towards one-step conversion of cellulose to ethanol. Enzyme Microb Technol 2007a;40:1291–9.

den Haan R, Rose SH, Lynd LR, van Zyl WH. Hydrolysis and fermentation of amorphous cellulose by recombinant *Saccharomyces cerevisiae*. Metab Eng 2007b;9:87–94.

Desvaux M. *Clostridium cellulolyticum*: model organism of mesophilic cellulolytic clostridia. FEMS Microbiol Rev 2005;29:741–64.

Doi RH, Tamaru Y. The *Clostridium cellulovorans* cellulosome: an enzyme complex with plant cell wall degrading activity. Chem Rec 2001;1:24–32.

Elkins JG, Raman B, Keller M. Engineered microbial systems for enhanced conversion of lignocellulosic biomass. Curr Opin Biotechnol 2010;21:657–62.

Fernandes AR, Mira NP, Vargas RC, Canelhas I, Sá-Correia I. *Saccharomyces cerevisiae* adaptation to weak acids involves the transcription factor Haa1p and Haa1P-regulated genes. Biochem Biophys Res Commun 2005;337:95-103.

Fierobe HP, Mingardon F, Mechaly A, Bélaïch A, Rincon MT, Pagès S, et al. Action of designer cellulosomes on homogeneous versus complex substrates. J Biol Chem 2005;280:16325–34.

Fujita Y, Takahashi S, Ueda M, Tanaka A, Okada H, Morikawa Y, et al. Direct and efficient production of ethanol from cellulosic material with a yeast strain displaying cellulolytic enzymes. Appl Environ Microbiol 2002;68:5136–41.

Fujita Y, Ito J, Ueda M, Fukuda H, Kondo A. Synergistic saccharification, and direct fermentation to ethanol of amorphous cellulose by use of engineered yeast strain codisplaying three types of cellulolytic enzyme. Appl Environ Microbiol 2004;70:1207–12.

Galazka JM, Tian C, Beeson WT, Martinez B, Glass NL, Cate JHD. Cellodextrin transport in yeast for improved biofuel production. Science 2010;330:84–6.

Gírio FM, Fonseca C, Carvalheiro F, Duarte LC, Marques S, Bogel-Lukasik R. Hemicelluloses for fuel ethanol: a review. Bioresour Technol 2010;101:4775–800.

Gnansounou E. Fuel ethanol. In: Pandey A, editor. Handbook of plant-based biofuels. CRC Press; 2008. p. 57–72.

Gong CS, Maun CM, Tsao GT. Direct fermentation of cellulose to ethanol by a cellulolytic filamentous fungus, *Monilla sp*. Biotechnol Lett 1981;3:77–82.

Gorsich SW, Dien BS, Nichols NN, Slininger PJ, Liu ZL, Skory CD. Tolerance to furfural-induced stress is associated with pentose phosphate pathway genes *ZWF1*, *GND1*, *RPE1*, and *TKL1* in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 2006;71:339–49.

Grabber JH. How do lignin composition, structure, and cross-linking affect degradability? A review of cell wall model studies. Crop Sci 2005;45:820–31.

Guldfeldt LU, Arneborg N. Measurement of the effects of acetic acid and extracellular pH on intracellular pH of nonfermenting, individual *Saccharomyces cerevisiae* cells by fluorescence microscopy. Appl Environ Microbiol 1998;64:530–4.

Hahn-Hägerdal B, Karhumaa K, Jeppsson M, Gorwa-Grauslund MF. Metabolic engineering for pentose utilization in *Saccharomyces cerevisiae*. Adv Biochem Eng Biotechnol 2007;108:147–77.

Hasunuma T, Sanda T, Yamada R, Yoshimura K, Ishii J, Kondo A. Metabolic pathway engineering based on metabolomics confers acetic and formic acid tolerance to a recombinant xylose-fermenting strain of *Saccharomyces cerevisiae*. Microb Cell Fact 2011a;10:2.

Hasunuma T, Sung KM, Sanda T, Yoshimura K, Matsuda F, Kondo A. Efficient fermentation of xylose to ethanol at high formic acid concentrations by metabolically engineered *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 2011b;90:997-1004.

Heer D, Heine D, Sauer U. Resistance of *Saccharomyces cerevisiae* to high concentrations of furfural is based on NADPH-dependent reduction by at least two oxidoreductase. Appl Environ Microbiol 2009;75:7631–8.
Heipieper HJ, Weber FJ, Sikkema J, Keweloh H, de Bont JAM. Mechanism of resistance of whole cells to toxic organic solvents. Trends Biotechnol 1994;12:409–15.
Hendriks ATWM, Zeeman G. Pretreatment to enhance the digestability of lignocellulosic biomass. Bioresour Technol 2009;100:10–8.
Herrmann MC, Vrsanska M, Jurickova M, Hirsch J, Biely P, Kubicek CP. The β-D-xylosidase of *Trichoderma reesei* is a multifunctional β-D-xylan xylohydrolase. Biochem J 1997;321:375–81.
Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, Brady JW, et al. Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 2007;315:804–7.
Himmel ME, Xu Q, Luo Y, Ding SY, Lamed R, Bayer EA. Microbial enzyme systems for biomass conversion: emerging paradigms. Biofuels 2010;1:323–41.
Hong J, Wang Y, Kumagai H, Tamaki H. Construction of thermotolerant yeast expressing thermostable cellulase genes. J Biotechnol 2007;130:114–23.
Ito J, Kosugi A, Tanaka T, Kuroda K, Shibasaki S, Ogino C, et al. Regulation of the display ratio of enzymes on the *Sacchromyces cerevisiae* cell surface by the immunoglobulin G and cellulosomal enzyme binding domains. Appl Environ Microbiol 2009;75:4149–54.
Jeffries TW. Utilization of xylose by bacteria, yeasts, and fungi. Adv Biochem Eng 1983;27:1-32.
Jeon E, Hyeon JE, Eun LS, Park BS, Kim SW, Lee J, et al. Cellulosic alcoholic fermentation using recombinant *Saccharomyces cerevisiae* engineered for the production of *Clostridium cellulovorans* endoglucanase and *Saccharomycopsis fibuligera* β-glucosidase. FEMS Microbiol Lett 2009;301:130–6.
Jin M, Balan V, Gunawan C, Dale BE. Consolidated bioprocessing (CBP) performance of *Clostridium phytofermentans* on AFEX-treated corn stover for ethanol production. Biotechnol Bioeng 2011;108:1290–7.
Karhumaa K, Sanchez RG, Hahn-Hägerdal B, Gorwa-Grauslund MF. Comparison of the xylose reductase-xylitol dehydrogenase and the xylose isomerase pathways for xylose fermentation by recombinant *Saccharomyces cerevisiae*. Microb Cell Fact 2007;6:5.
Katahira S, Fujita Y, Mizukie A, Fukuda H, Kondo A. Construction of a xylan fermenting yeast strain through codisplay of xylanolytic enzymes on the surface of xylose utilizing *Saccharomyces cerevisiae* cells. Appl Environ Microbiol 2004;70:5407–14.
Katahira S, Mizuike A, Fukuda H, Kondo A. Ethanol fermentation from lignocellulosic hydrolysate by a recombinant xylose- and cellooligosaccharide-assimilating yeast strain. Appl Microbiol Biotechnol 2006;72:1136–43.
Khan QA, Hadi SA. Inactivation and repair of bacteriophage lambda by furfural. Biochem Mol Biol Int 1994;32:379–85.
Kitamoto N, Yoshino S, Ohmiya K, Tsukagoshi N. Sequence analysis, overexpression, and antisense inhibition of a β-xylosidase gene, *xylA*, from *Aspergillus oryzae* KBN616. Appl Environ Microbiol 1999;65:20–4.
Klinke HB, Olsson L, Thomsen AB, Ahring BK. Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of *Saccharomyces cerevisiae*: wet oxidation and fermentation by yeast. Biotechnol Bioeng 2003;81:738–47.
Klinke HB, Thomsen AB, Ahring AB. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl Microbiol Biotechnol 2004;66:10–26.
Kondo A, Ueda M. Yeast cell-surface display-application of molecular display. Appl Microbiol Biotechnol 2004;64:28–40.
Kondo A, Shigechi H, Abe M, Uyama K, Matsumoto T, Takahashi S, et al. High level production from starch by a flocculent *Saccharomyces cerevisiae* strain displaying cell surface glucoamylase. Appl Microbiol Biotechnol 2002;58:291–6.
Kotaka A, Bando H, Kaya M, Kato-Murai M, Kuroda K, Sahara H, et al. Direct ethanol production from barley β-glucan by sake yeast displaying *Aspergillus oryzae* β-glucosidase and endoglucanase. J Biosci Bioeng 2008;105:622–7.
Kulkarni N, Shendye A, Rao M. Molecular and biotechnological aspects of xylanases. FEMS Microbiol Rev 1999;23:411–56.
la Grange DC, Pretorius IS, van Zyl WH. Expression of a *Trichoderma reesei* β-xylanase gene (*XYN2*) in *Saccharomyces cerevisiae*. Appl Environ Microbiol 1996;62:1036–44.
la Grange DC, Pretorius IS, van Zyl WH. Cloning of the *Bacillus pumilus* β-xylosidase gene (*xynB*) and its expression in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 1997;47:262–6.
la Grange DC, Pretorius IS, Claeyssens M, van Zyl WH. Degradation of xylan to D-xylose by recombinant *Saccharomyces cerevisiae* coexpressing the *Aspergillus niger* β-xylosidase (*xlnD*) and the *Trichoderma reesei* xylanase II (*xyn2*) genes. Appl Environ Microbiol 2001;67:5512–9.
la Grange DC, den Haan R, van Zyl WH. Engineering cellulolytic ability into bioprocessing organisms. Appl Microbiol Biotechnol 2010;87:1195–208.
Laadan B, Almeida JR, Rådström P, Hahn-Hägerdal B, Gorwa-Grauslund M. Identification of an NADH-dependent 5-hydroxymethylfurfural-reducing alcohol dehydrogenase in *Saccharomyces cerevisiae*. Yeast 2008;25:191–8.
Larsson S, Reimann A, Nilvebrant NO, Jönsson LJ. Comparison of different methods for the detoxification of lignocellulose hydrolysates of spruce. Appl Biochem Biotechnol 1999;77:91-104.
Larsson S, Quintana-Sáinz A, Reimann A, Nilvebrant N-O, Jönsson LJ. Influence of lignocellulose-derived aromatic compounds on oxygen-limited growth and ethanolic fermentation by *Saccharomyces cerevisiae*. Appl Biochem Biotechnol 2000;84–86:617–32.
Larsson S, Cassland P, Jönsson LJ. Development of a *Saccharomyces cerevisiae* strain with enhanced resistance to phenolic fermentation inhibitors in lignocellulose hydrolysates by heterologous expression of laccase. Appl Environ Microbiol 2001a;67:1163–70.
Larsson S, Nilvebrant N-O, Jönsson LJ. Effect of overexpression of *Saccharomyces cerevisiae* Pad1p on the resistance to phenylacrylic acids and lignocellulose hydrolysates under aerobic and oxygen-limited conditions. Appl Microbiol Biotechnol 2001b;57:167–74.
Lau MW, Gunawan C, Balan V, Dale BE. Comparing the fermentation performance of *Escherichia coli* KO11, *Saccharomyces cerevisiae* 424A (LNH-ST) and *Zymomonas mobilis* AX101 for cellulosic ethanol production. Biotechnol Biofuels 2010;3:11.
Li XL, Lijungdahl LG. Expression of *Aureobasidium pullulans xynA* in, and secretion of the xylanase from, *Saccharomyces cerevisiae*. Appl Environ Microbiol 1996;62:209–13.
Li BZ, Yuan YJ. Transcriptome shifts in response to furfural and acetic acid in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 2010;86:1915–24.
Lilly M, Fierobe HP, van Zyl WH, Volschenk H. Heterologous expression of a *Clostridium* minicellulosome in *Saccharomyces cerevisiae*. FEMS Yeast Res 2009;9:1236–49.
Lin FM, Qiao B, Yuan YJ. Comparative proteomic analysis of tolerance and adaptation of ethanologenic *Saccharomyces cerevisiae* to furfural, lignocellulosic inhibitory compound. Appl Environ Microbiol 2009;75:3765–76.
Linger JG, Adney WS, Darzins A. Heterologous expression and extracellular secretion of cellulolytic enzymes by *Zymomonas mobilis*. Appl Environ Microbiol 2010;76:6360–9.
Liu ZL. Genomic adaptation of ethanologenic yeast to biomass conversion inhibitors. Appl Microbiol Biotechnol 2006;73:27–36.
Liu ZL. Molecular mechanism of yeast tolerance and in situ detoxification of lignocellulosic hydrolysates. Appl Microbiol Biotechnol 2011;90:809–25.
Liu ZL, Blaschek HP. Lignocellulosic biomass conversion to ethanol by *Saccharomyces*. In: Vertes A, Qureshi N, Yukawa H, Blaschek H, editors. Biomass to biofuels. West Sussex: Wiley; 2009. p. 17–36.
Liu ZL, Moon J. A novel NADPH-dependent aldehyde reductase gene from *Saccharomyces cerevisiae* NRRL Y-12632 involved in the detoxification of aldehyde inhibitors derived from lignocellulosic biomass conversion. Gene 2009;446:1-10.
Liu ZL, Slininger PJ, Dien BS, Berhow MA, Kurtzman CP, Gorsich SW. Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethylfuran. J Ind Microbiol Biotechnol 2004;31:345–52.
Liu ZL, Moon J, Andersh BJ, Slininger PJ, Weber S. Multiple gene-mediated NAD(P)H-dependent aldehyde reduction is a mechanism of in situ detoxification of furfural and 5-hydroxymethylfurfural by *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 2008;81:743–53.
Liu ZL, Ma M, Song M. Evolutionarily engineered ethanologenic yeast detoxifies lignocellulosic biomass conversion inhibitors by reprogrammed pathways. Mol Genet Genomics 2009;282:233–44.
Lu Y, Zhang YHP, Lynd LR. Enzyme-microbe synergy during cellulose hydrolysis by *Clostridium thermocellum*. Proc Natl Acad Sci USA 2006;103:16165–9.
Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 2002;66:506–77.
Lynd LR, van Zyl WH, McBride JE, Laser M. Consolidated bioprocessing of cellulosic biomass: an update. Curr Opin Biotechnol 2005;16:577–83.
Ma M, Liu ZL. Comparative transcriptome profiling analyses during the lag phase uncover *YAP1*, *PDR1*, *PDR3*, *RPN4*, and *HSF1* as key regulatory genes in genomic adaptation to the lignocellulose derived inhibitor HMF for *Saccharomyces cerevisiae*. BMC Genomics 2010;11:660.
Madshus IH. Regulation of intracellular pH in eukaryotic cells. Biochem J 1988;250:1–8.
Margolles-Clark E, Tenkanen M, Nakari-Setälä T, Penttilä M. Cloning of genes encoding α-L-arabinofuranosidase and β-xylosidase from *Trichoderma reesei* by expression in *Saccharomyces cerevisiae*. Appl Environ Microbiol 1996;62:3840–6.
Martin C, Alriksson B, Sjöde A, Nilvebrant NO, Jönsson LJ. Dilute sulfuric acid pretreatment of agricultural and agro-industrial residues for ethanol production. Appl Biochem Biotechnol 2007;137:339–52.
Martín C, Marcet M, Almazán O, Jönsson LJ. Adaptation of a recombinant xylose-utilizing *Saccharomyces cerevisiae* to a sugarcane bagasse hydrolysate with high content of fermentation inhibitors. Bioresour Technol 2007;98:1767–73.
Medve J, Ståhlberg J, Tjerneld F. Adsorption and synergism of cellobiohydrolase I and II of *Trichoderma reesei* during hydrolysis of microcrystalline cellulose. Biotechnol Bioeng 1994;44:1064–73.
Medve J, Karlsson J, Lee D, Tjerneld F. Hydrolysis of microcrystalline cellulose by cellobiohydrolase I and endoglucanase II from *Trichoderma reesei*: adsorption, sugar production pattern, and synergism of the enzymes. Biotechnol Bioeng 1998;59:621–34.
Mira NP, Palma M, Guerreiro JF, Sá-Correia I. Genome-wide identification of *Saccharomyces cerevisiae* genes required for tolerance to acetic acid. Microb Cell Fact 2010a;9:79.
Mira NP, Teixeira MC, Sá-Correia I. Adaptive response and tolerance to weak acids in *Saccharomyces cerevisiae*: a genome-wide view. OMICS 2010b;14:525–40.
Modig T, Liden G, Taherzadeh MJ. Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase. Biochem J 2002;363:769–76.
Moreau A, Durand S, Morosoli R. Secretion of a *Cryptococcus albidus* xylanase in *Saccharomyces cerevisiae*. Gene 1992;116:109–13.
Morosoli R, Zalce E, Durand S. Secretion of a *Cryptococcus albidus* xylanase in *Pichia stipitis* resulting in a xylan fermenting transformant. Curr Genet 1993;24:94–9.
Mroczek S, Kufel J. Apoptic signals induce specific degradation of ribosomal RNA in yeast. Nucleic Acids Res 2008;36:2874–88.
Navarro AR. Effects of furfural on ethanol fermentation by *Saccharomyces cerevisiae*: mathematical models. Curr Microbiol 1994;29:87–90.
Nevoigt E. Progress in metabolic engineering of *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev 2008;72:379–412.
Olsson L, Hahn-Hägerdal B. Fermentative performance of bacteria and yeasts in lignocellulose hydrolysates. Process Biochem 1993;28:249–57.

Palmqvist E, Hahn-Hägerdal B. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresour Technol 2000;74:25–33.
Palmqvist E, Almeida JS, Hahn-Hägerdal B. Influence of furfural on anaerobic glycolytic kinetics of *Saccharomyces cerevisiae* in batch culture. Biotechnol Bioeng 1999;62: 447–54.
Pampulha ME, Loureiro-Dias MC. Activity of glycolytic enzymes of *Saccharomyces cerevisiae* in the presence of acetic acid. Appl Microbiol Biotechnol 1990;34:375–80.
Panagiotou G, Villas-Boas SG, Christakopoulos P, Nielsen J, Olsson L. Intracellular metabolite profiling of *Fusarium oxysporum* converting glucose to ethanol. J Biotechnol 2005;115:425–34.
Pérez-Gonzalez JA, De Graff LH, Visser J, Ramón D. Molecular cloning and expression in *Saccharomyces cerevisiae* of two *Aspergillus nidulans* xylanase genes. Appl Environ Microbiol 1996;62:2179–82.
Petersson A, Almeida JR, Modig T, Karhumaa K, Hahn-Hägerdal B, Gorwa-Grauslund MF, et al. A 5-hydroxymethyl furfural reducing enzyme encoded by the *Saccharomyces cerevisiae ADH6* gene conveys HMF tolerance. Yeast 2006;23:455–64.
Piper P, Mahé Y, Thompson S, Pandjaitan R, Holyoak C, Egner R, et al. The Pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast. EMBO J 1998;17:4257–65.
Raman B, Pan C, Hurst GB, Rodriguez Jr M, McKeown CK, Lankford PK, et al. Impact of pretreated switchgrass and biomass carbohydrates on *Clostridium thermocellum* ATCC 27405 cellulosome composition: a quantitative proteomic analysis. PLoS One 2009;4:e5271.
Ruiz E, Romero I, Moya M, Sanchez S, Bravo V, Castro E. Sugar fermentation by *Fusarium oxysporum* to produce ethanol. World J Microbiol Biotechnol 2007;23:259–67.
Russell JB. Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. J Appl Bacteriol 1992;73:363–70.
Ryabova OB, Chmil OM, Sibirny AA. Xylose and cellobiose fermentation to ethanol by the thermotolerant methylotrophic yeast *Hansenula polymorpha*. FEMS Yeast Res 2003;4:157–64.
Ryu S, Karim MN. A whole cell biocatalyst for cellulosic ethanol production from dilute acid-pretreated corn stover hydrolyzates. Appl Microbiol Biotechnol 2011;91: 529–42.
Saha BC. Hemicellulose bioconversion. J Ind Microbiol Biotechnol 2003;30:279–91.
Sakamoto T, Hasunuma T, Hori Y, Yamada R, Kondo A. Direct ethanol production from hemicellulosic materials of rice straw by use of an engineered yeast strain codisplaying three types of hemicellulolytic enzymes on the surface of xylose-utilizing *Saccharomyces cerevisiae* cells. J Biotechnol 2011. doi:10.1016/j.biotec.2011.06.025.
Sanchez B, Bautista J. Effects of furfural and 5-hydroxymethylfurfural on the fermentation of *Saccharomyces cerevisiae* and biomass production from *Candida guilliermondii*. Enzyme Microb Technol 1988;10:315–8.
Sánchez OJ, Cardona CA. Trends in biotechnological production of fuel ethanol from different feedstocks. Bioresour Technol 2008;99:5270–95.
Sanda T, Hasunuma T, Matsuda F, Kondo A. Repeated-batch fermentation of lignocellulosic hydrolysate to ethanol using a hybrid *Saccharomyces cerevisiae* strain metabolically engineered for tolerance to acetic and formic acid. Bioresour Technol 2011. doi:10.1016/j.biortech.2011.06.028.
Sauer U. Evolutionary engineering of industrially important microbial phenotypes. Adv Biochem Eng Biotechnol 2001;73:129–69.
Shoham Y, Lamed R, Bayer EA. The cellulosome concept as an efficient microbial strategy for the degradation of insoluble polysaccharides. Trends Microbiol 1999;7: 275–81.
Skory CD, Freer SN, Bothast RJ. Screening for ethanol-producing filamentous fungi. Biotechnol Lett 1997;19:203–6.
Slininger PJ, Thompson SR, Weber S, Liu ZL, Moon J. Repression of xylose-specific enzymes by ethanol in *Scheffersomyces* (*Pichia*) *stipitis* and utility of repitching xylose-grown populations to eliminate diauxic lag. Biotechnol Bioeng 2011;108: 1801–15.
Sonderegger M, Sauer U. Evolutionary engineering of *Saccharomyces cerevisiae* for anaerobic growth on xylose. Appl Environ Microbiol 2003;69:1990–8.
Stevenson DM, Weimer PJ. Isolation and characterization of a *Trichoderma* strain capable of fermenting cellulose to ethanol. Appl Microbiol Biotechnol 2002;59:721–6.
Sticker AR, Mach RL, de Graaff LH. Regulation of transcription of cellulases- and hemicellulases-encoding genes in *Aspergillus niger* and *Hypocrea jecorina* (*Trichoderma reesei*). Appl Microbiol Biotechnol 2008;78:211–20.
Sundström L, Larsson S, Jönsson LJ. Identification of *Saccharomyces cerevisiae* genes involved in the resistance to phenolic fermentation inhibitors. Appl Biochem Biotechnol 2010;161:106–15.
Takada G, Kawaguchi T, Sumitani J, Arai M. Expression of *Aspergillus aculeatus* No. F-50 cellobiohydrolase I (*cbhI*) and β-glucosidase 1 (*bgl1*) genes by *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem 1998;62:1615–8.
Tanaka A, Ueda M. Cell surface engineering of yeast: construction of arming yeast with biocatalyst. J Biosci Bioeng 2000;90:125–36.
Teeri TT. Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. Trends Biotechnol 1997;15:160–7.
Thomsen MH, Thygesen A, Thomsen AB. Identification and characterization of fermentation inhibitors formed during hydrothermal treatment and following of wheat straw. Appl Microbiol Biotechnol 2009;83:447–55.
Tomás-Pejó E, Ballesteros M, Oliva JM, Olsson L. Adaptation of the xylose fermenting yeast *Saccharomyces cerevisiae* F12 for improving ethanol production in different fed-batch SSF processes. J Ind Microbiol Biotechnol 2010;37:1211–20.
Törrönen A, Harkki A, Rouvinen J. Three-dimensional structure of endo-1,4-β-xylanase II from *Trichoderma reesei*: two conformational states in the active site. EMBO J 1994;13:2493–501.
Tsai SL, Oh J, Singh S, Chen R, Chen W. Functional assembly of minicellulosomes on the *Sacchromyces cerevisiae* cell surface for cellulose hydrolysis and ethanol production. Appl Environ Microbiol 2009;75:6087–93.
Tsai SL, Goyal G, Chen W. Surface display of a functional minicellulosome by intracellular complementation using a synthetic yeast consortium and its application to cellulose hydrolysis and ethanol production. Appl Environ Microbiol 2010;76: 7514–20.
van Maris AJA, Abbott DA, Bellissimi E, van den Brink J, Kuyper M, Luttik MAH, et al. Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. Antonie Van Leeuwenhoek 2006;90:391–418.
van Rensburg P, van Zyl WH, Pretorius IS. Engineering yeast for efficient cellulose degradation. Yeast 1998;14:67–76.
van Rooyen R, Hahn-Hägerdal B, la Grange DC, van Zyl WH. Construction of cellobiose-growing and fermenting *Sacchromyces cerevisiae* strains. J Biotechnol 2005;120:284–95.
van Vleet JH, Jeffries TW. Yeast metabolic engineering for hemicellulosic ethanol production. Curr Opin Biotechnol 2009;20:300–6.
van Zyl WH, Lynd LR, den Haan R, McBride JE. Consolidated bioprocessing for bioethanol production using *Saccharomyces cerevisiae*. Adv Biochem Eng Biotechnol 2007;108:205–35.
Vasan PT, Piriya PS, Prabhu DI, Vennison SJ. Cellulosic ethanol production by *Zymomonas mobilis* harboring an endoglucanase gene from *Enterobacter cloacae*. Bioresour Technol 2011;102:2585–9.
Vinzant TB, Adney WS, Decker SR, Baker JO, Kinter MT, et al. Fingerprinting *Trichoderma reesei* hydrolases in a commercial cellulase preparation. Appl Biochem Biotechnol 2001;91:99-107.
Voronovsky AY, Rohuya OV, Abbas CA, Sibirny AA. Development of strains of the thermotolerant yeast *Hansenula polymorpha* capable of alcoholic fermentation of starch and xylan. Metab Eng 2009;11:234–42.
Wahlbom CF, van Zyl WH, Jönsson LJ, Hahn-Hägerdal B, Otero RRC. Generation of the improved recombinant xylose-utilizing *Saccharomyces cerevisiae* TMB 3400 by random mutagenesis and physiological comparison with *Pichia stipitis* CBS 6054. FEMS Yeast Res 2003;3:319–26.
Wen F, Sun J, Zhao H. Yeast surface display of trifunctional minicellulosomes for simultaneous saccharification and fermentation of cellulose to ethanol. Appl Environ Microbiol 2010;76:1251–60.
Wisselink HW, Toirkens MJ, Wu Q, Pronk JT, van Maris AJA. Novel evolutionary engineering approach for accelerated utilization of glucose, xylose, and arabinose mixtures by engineered *Saccharomyces cerevisiae* strains. Appl Environ Microbiol 2009;75:907–14.
Wood TM. Fungal cellulases. Biochem Soc Trans 1992;20:46.
Woodward J. Synergism in cellulose systems. Bioresour Technol 1991;36:67–75.
Xu Q, Singh A, Himmel ME. Perspectives and new directions for the production of bioethanol using consolidated bioprocessing of lignocellulose. Curr Opin Biotechnol 2009;20:364–71.
Xu C, Qin Y, Li Y, Ji Y, Huang J, Song H, et al. Factors influencing cellulosome activity on Consolidated Bioprocessing of cellulosic ethanol. Bioresour Technol 2010;101: 9560–9.
Yamada R, Taniguchi N, Tanaka T, Ogino C, Fukuda H, Kondo A. Cocktail δ-integration: a novel method to construct cellulolytic enzyme expression ratio-optimized yeast strain. Microb Cell Fact 2010;9:32.
Yamada R, Taniguchi N, Tanaka T, Ogino C, Fukuda H, Kondo A. Direct ethanol production from cellulosic materials using a diploid strain of *Saccharomyces cerevisiae* with optimized cellulase expression. Biotechnol Biofuels 2011;4:8.
Yanase S, Hasunuma T, Yamada R, Tanaka T, Ogino C, Fukuda H, et al. Direct ethanol production from cellulosic materials at high temperature using the thermotolerant yeast *Kluyveromyces marxianus* displaying cellulolytic enzymes. Appl Microbiol Biotechnol 2010a;88:381–8.
Yanase S, Yamada R, Kaneko S, Noda H, Hasunuma T, Tanaka T, et al. Ethanol production from cellulosic materials using cellulase-expressing yeast. Biotechnol J 2010b;5:449–55.
Zhang YH, Lynd LR. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. Biotechnol Bioeng 2004;88:797–824.
Zhang YHP, Himmel ME, Mielenz JR. Outlook for cellulase improvement: screening and selection strategies. Biotechnol Adv 2006;24:452–81.
Zhang J, Zhu Z, Wang X, Wang N, Wang W, Bao J. Biodetoxification of toxins generated from lignocellulose pretreatment using a newly isolated fungus, *Amorphotheca resinae* ZN1, and the consequent ethanol production. Biotechnol Biofuels 2010;3: 26.
Zhang JG, Liu XY, He XP, Guo XN, Lu Y, Zhang B. Improvement of acetic acid tolerance and fermentation performance of *Saccharomyces cerevisiae* by disruption of the *FPS1* aquaglyceroporin gene. Biotechnol Lett 2011;33:277–84.
Zheng DQ, Wu XC, Tao XL, Wang PM, Li P, Chi XQ, et al. Screening and construction of *Saccharomyces cerevisiae* strains with improved multi-tolerance and bioethanol fermentation performance. Bioresour Technol 2011;102:3020–7.
Zhou S, Ingram LO. Simultaneous saccharification and fermentation of amorphous cellulose to ethanol by recombinant *Klebsiella oxytoca* SZ21 without supplemental celloulase. Biotechnol Lett 2001;23:1455–62.
Zhou S, Davis FC, Ingram LO. Gene integration and expression and extracellular secretion of *Erwinia chrysanthemi* endoglucanase CelY (*celY*) and CelZ (*celZ*) in ethanologenic *Klebsiella oxytoca* P2. Appl Environ Microbiol 2001;67:6-14.