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Improved Ethanol and Reduced Xylitol Production from Glucose and Xylose Mixtures by the Mutant Strain of *Candida shehatae* ATCC 22984

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Abstract A mutant Cs3512, which showed better fermentation of xylose and the mixtures of xylose and glucose, was obtained through mutation of *Candida shehatae* ATCC 22984 and screening with a medium containing antimycin A and TTC (2,3,5-triphenyltetrazolium chloride). Cs3512 produced 44.4 g/l of ethanol from 121.3 g/l of xylose, which was 13% higher than that by ATCC 22984. At the same time, xylitol production was reduced by 38% to 10.2 g/l from 16.3 g/l by ATCC 22984. Cs3512 also showed 8% increase in ethanol yield from 0.39 to 0.42 g/g comparing to ATCC 22984 when fermenting the sugar mixture composed of 52.9 g/l glucose and 21.2 g/l xylose. When Cs3512 was used in the simultaneous saccharification and fermentation of lime pretreated rice straw via CaCCO (calcium capturing by carbonation) process, it produced ethanol at 77% of the theoretical yield. The results imply that Cs3512 is a potential non-recombinant yeast strain for ethanol production from lignocellulosic biomass.

Keywords *Candida shehatae* · Mutation · Xylose · SSF · Rice straw

Introduction

Fuel-ethanol (bioethanol) production from the abundant lignocellulosic resources, called the second generation of bioethanol, has attracted considerable attention in recent years because

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of the high cost of raw materials (foodstuffs) and the competition with food of human being and livestock in the traditional ethanol industry [1–3].

Xylose is the secondary abundant fermentable sugar in the hydrolysate of lignocellulosic biomass [4, 5]. Therefore, it is important to utilize xylose efficiently along with the fermentation of glucose for the economical ethanol production from lignocellulose, which is different from the glucose fermentation-based traditional ethanol production processes [6, 7]. However, there are only a few types of yeasts, such as *Candida shehatae*, *Pichia stipitis*, and *Pachysolen tannophilus*, which can efficiently ferment both glucose and xylose to ethanol [8]. On the other hand, the fermentation of sugar mixtures by recombinant microorganisms has achieved considerable progress in recent years. However, due to the public concern of the biohazard, the large-scale application of recombinant microorganisms still faces some limitations [9]. Therefore, improving the fermentation ability of wild yeasts by non-recombinant methods such as mutation attracted many researchers [10, 11]. The mutation and screen processes have already been successfully used to improve the performance of many industrial-using microorganisms such as *Penicillium notatum* and *Micromonospora echinospora* [12]. In the past few decades, it has also been used to overcome the disadvantages of wild xylose-fermenting yeasts for ethanol production [11, 13, 14].

Among the wild xylose-fermenting yeasts, *C. shehatae* is a good candidate for sugar mixtures fermentation because it can ferment variant ratios of glucose and xylose to ethanol at high specific rate (0.55–2.20 g/g/l), comparing with *P. stipitis* (0.11–0.69 g/g/l) and *P. tannophilus* (0.05–0.76 g/g/l), as well as with relatively high ethanol yield (0.35–0.41 g/g) [15]. However, like most of the wild xylose-fermenting yeasts, *C. shehatae* not only produces ethanol inefficiently at high aeration condition but also produces much amount of xylitol at low oxygen condition [16] or in the presence of glucose [15]. At the same time, the overall ethanol production from lignocellulosic biomass by *C. shehatae* was relatively low [17].

In this research, a high-ethanol-yield mutant from *C. shehatae* ATCC 22984 was obtained by our mutation strategy. This mutant also gained another advantage over ATCC22984 with less xylitol production when fermenting either xylose or the mixtures of glucose and xylose. In addition, the mutant showed feasibility of producing ethanol from the rice straw that was pretreated with a newly developed CaCCO (calcium capturing via carbonation) process [18] via simultaneous saccharification and fermentation (SSF).

Materials and Methods

The yeast *C. shehatae* ATCC 22984 was obtained from The American Type Culture Collection. The strain was stored on YPM-X plates at 4 °C, which were composed of YPM (per liter: Bacto peptone 5 g, Bacto yeast extract 3 g, Bacto malt extract 3 g, $CaCl_2$ 200 mg, KH_2PO_4 2.5 g, $MgSO_4 \cdot 7H_2O$ 500 mg, and $(NH_4)_2SO_4$ 1 g), 10 g/l of xylose, and 20 g/l of Bacto agar. It was grown in YPM-2X (YPM with 20 g/l of xylose) medium at 30 °C and 250 rpm for 24 h before harvest.

Mutation

The mutation was induced by UV irradiation (GL 15, Toshiba Lighting and Technology Co. Ltd., Yokosuka, Japan) in a clean bench (MCV-131BMS, SANYO, Osaka, Japan).

The 24-h pre-cultivated yeast was separated by centrifugation (high-speed refrigerated centrifuge CR22G II, HITACH, Tokyo, Japan) at 6,000×*g* for 5 min, and the pellet was re-suspended in saline to make the cells concentration in the suspension to be 0.2 dry weight g/ml (tested as described below). A fraction of the suspension (20 ml) was transferred to a 20-cm culture dish (without cover) and was exposed to UV light while being stirred on a stirrer (CT-1A, AS ONE Co. Ltd., Osaka, Japan). The irradiation continued for 35 s to obtain the survival rate of 5% (Eq. 1). The sample of 100 μL was transferred to a 1.5-ml tube and then was chilled in ice for 2 h keeping away from light. After chilling, 1 ml of YPM-X medium was added to a tube, and then the mixture was cultivated at 30 °C for another 2 h for reactivation. Following that, the medium was diluted to proper cell concentrations with distilled water and was spread onto the YPM-X plate containing 0.05 g/l of antimycin A (Wako Pure Chemical Industrials, Osaka, Japan) [11]. The plate was cultivated at 25 °C for 2 days for the growth of cells. The survival rate was calculated as Eq. 1.

$$\%\ \text{Survival rate} = \frac{\text{Colonies in the plate inoculated by the cells irradiated for certain time}}{\text{Colonies in the plate inoculated by the cells without irradiation}} \times 100\% \tag{1}$$

The grown colonies were stained by a lay of TTC agar (10 ml) which was composed of 0.5 g/l of xylose, 10 g/l of agar, and 0.05 g/l of 2,3,5-triphenyltetrazolium chloride (TTC) [19]. The TTC agar-covered plate was further cultivated at 25 °C for 2 h for color development. The colonies that showed dark red color were selected for ethanol production evaluation.

The mutation and selection experiments were executed for two rounds. In the first round, the selected strains were compared by fermentation using the YPM-2X medium (5 ml per tube) in the centrifugation tubes sealed by a cap without aeration. The tubes were cultivated at 30 °C and 250 rpm for 24 h in the Bio-shaker (BR-23FP, TAITEC, Tokyo, Japan). The strain showing the best ethanol production was used for the second round of mutation and selection. In the second round, the selected strains were compared by fermentation using the YPM-5G2X medium (YPM plus 50 g/l of glucose and 20 g/l of xylose, 5 ml per tube) in the culture tubes (filter tube 50, TPP AG, Trasadingen, Switzerland). The culture tube was sealed by a cap with five holes (Hole-A to Hole-E; the holes were sealed with 0.45-μm membrane). During the cultivation, the holes except Hole-A were sealed with tape to control the strength of aeration. The culture tubes were cultivated at 30 °C and 250 rpm for 24 h in the Bio-shaker. The strain that showed the best fermentation potential (named as Cs3512) was selected for further characterization.

Pretreatment of Rice Straw

The sun-dried rice straw (cv. *Koshihikari*) used in this research was purchased from Honda Noen Co. Ltd. (Tsukubamirai, Ibaraki, Japan). Before pretreatment, it was further dried at 70 °C for 3 days and milled to 0.5 mm by a high-speed milling machine (PM-2005, Osaka Chemical, Osaka, Japan). The milled rice straw was composed of 31.5% glucan, 14.5% xylan, 2.5% arabinose, 1.3% galactose, 16.8% lignin, 21.7% ash, and 4.6% water (*w*/*w*), which was analyzed by the methods described before [20].

The milled rice straw was pretreated by heating with lime and water (mixed with the ratio of 10:2:90, based on weight) at 120 °C for 1 h in a high-pressure steam sterilizer (LSX-700, TOMY, Tokyo, Japan). After cooling down to room temperature, the mixture was further

milled five times with a milling machine (micro powder MPW-G008, WEST Co. Ltd., Niigata, Japan). Then, the pretreated mixture (10 g) was transferred to a serum bottle (50 ml, sealed by a butyl-rubber stopper) and neutralized by injection with CO_2 (0.11 MPa, 30 min). The final pH of the mixture was 6.5. There was no washing and separation process in this CO_2 neutralization process. After neutralization, the mixture was directly used for fermentation via SSF without detoxification and nutrients replenishment.

Fermentation of Sugars in Synthetic Medium

Culture tubes (filter tube 50) were used as the reactors to evaluate the mutants and their parent strain ATCC 22984. The 24-h pre-cultivated yeast cells were inoculated to 10 ml of fresh medium for fermentation. The initial concentration of cells was adjusted to 0.33 dry weight g/ml (tested as described below). In fermentation, all the holes in the cap of the tube except Hole-A were sealed by tape. The tubes were cultivated at 30 °C, 250 rpm in a shaking incubator (BR-23FP Bioshaker). Samples were taken out at setting interval for analysis.

Simultaneous Saccharification and Fermentation of Lime-Pretreated Rice Straw

The pretreated rice straw (10 g) was fermented in the serum bottle (50 ml) which was sealed by a butyl-rubber stopper. The serum bottle was connected to the atmosphere through a syringe needle (18 G×1.5′, TERUMO Co. Ltd., Tokyo, Japan) that was penetrated through the rubber stopper. To prevent contamination, the open end of the needle was sealed by a 0.2-μm filter (Millex-GV, Millipore, Crrigtwohill Co., Cork, Ireland). The SSF was started by addition of enzyme mixtures, which contained Celluclast 1.5 L (Novozymes Japan Co. Ltd., Chiba, Japan) as cellulase/hemicellulose [6.5 filter paper degradation unit (FPU) and 10 U xylanase (for birchwood xylan) per gram rice straw at pH 4.8], Novozyme 188 (Sigma-Aldrich Japan K.K., Chiba, Japan) as β-glucosidase [11 cellobiase unit (CbU) per gram rice straw at pH 4.8], Ultraflo L (Novozymes Japan) as hemicellulose/β-glucosidase [4.5 U xylanase and 0.5 CbU per gram rice straw at pH 4.8], and 200 μl of Cs3512 inoculum (to the final concentration of 0.22 dry weight g/ml). The bottle was cultivated at 30 °C, 800 rpm in the shaker (M·BR-022UP Bioshaker, TAITEC, Tokyo, Japan). Samples were taken by syringe to analyze the concentration of glucose, xylose, xylitol, glycerol, and ethanol.

Analysis Methods

The components of rice straw were analyzed according to the methods introduced elsewhere [20]. The concentration of microorganisms was measured by the optical density at 600 nm (OD_{600}) in 1-cm path length cells by a spectrometer (SpectraMax Plus384, Molecular Devices, Tokyo, Japan). The culture which has an optical density OD_{600} of 1 contains 0.22 g/l of dry *C. shehatae* cells (dried at 105 °C to a constant weight). The concentration of glucose, xylose, xylitol, glycerol, acetate, and ethanol were measured by high-performance liquid chromatography (HPLC) using a Shimadzu LC-20 system (SHIMADZU Co. Ltd., Tokyo, Japan) with a RI detector (RID-10A, SHIMADZU Co. Ltd.) and an Aminex HPX-87 ion exclusion column (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The column temperature was 50 °C. The elution liquid was 5 mM of sulfuric acid solution, and the flow rate was 0.6 ml/min. The ethanol yield was defined as the ratio of produced ethanol (gram) divided by consumed

sugars (gram). The overall ethanol conversion efficiency was calculated using Eq. 2, and the specific ethanol production rate was calculated using Eq. 3.

$$\%\ \text{Overall ethanol yield efficiency} = \frac{\text{Ethanol produced (g)}}{\text{The amount of glucose and xylose in the rice straw (g)} \times 0.511} \times 100 \quad (2)$$

$$\text{Specific ethanol production rate (g/l/h)} = \frac{\text{Ethanol produced (g)}}{\text{Volume of culture medium (l)} \times \text{fermentation time (h)}} \quad (3)$$

All the experiments were run in duplicate, and the average value was used in this paper.

Results and Discussion

Mutation of *C. shehatae* ATCC 22984

Two rounds of mutation and screening were carried out to select the mutants that could produce more ethanol from glucose and xylose as well as less xylitol than the original strain. In the first round, 60 mutants which appeared dark red (the others showed white or pink) were selected from 10,000 colonies. These 60 mutants were tested for ethanol production in YPM-2X medium in tube reactors without aeration. Among the mutants, six strains surpassed ATCC 22984 in both specific ethanol production rate and ethanol yield (data not shown). Finally, a mutant referred as Cs35, which produced ethanol faster (26% improvement in specific ethanol production rate) and more efficient (10% improvement in ethanol yield) than the original strain ATCC 22984 (data not shown), was selected for the next round of mutation.

In the second round, 19 mutants which showed dark red were selected from 5,000 colonies. Their capability of producing ethanol from the sugar mixture (50 g/l glucose and 20 g/l xylose) was evaluated. Among them, Cs3512 was the most productive strain, which showed 5% improvement in both specific ethanol production rate and ethanol yield than Cs35 (data not shown). Then, Cs3512 was further researched to compare with ATCC 22984 and was applied to the fermentation of rice straw via SSF.

Fermentation of Sugars in Synthetic Medium

The capacity of Cs3512 to ferment glucose or xylose alone was evaluated firstly. The strain Cs3512 and ATCC 22984 fermented glucose at similar rate (Fig. 1). Finally, 46.4 and 46.3 g/l of ethanol were produced from glucose within 48 h by ATCC 22984 and Cs3512, respectively. This result indicated that the glucose fermentation ability was not severely affected by the mutation in Cs3512. The final ethanol concentration obtained in this study was close to the highest ethanol concentration (45–50 g/l) that could be achieved by *C. shehatae* [21]. This limitation was ascribed to the relatively low ethanol tolerance of *C. shehatae* [8]. Although the final ethanol concentration achieved by Cs3512 was similar with that of ATCC 22984, its ethanol yield was slightly higher than that of ATCC 22984. The ethanol yield increased from 0.38 g/g (by ATCC 22984) to 0.40 g/g (by Cs3512). This improvement might be

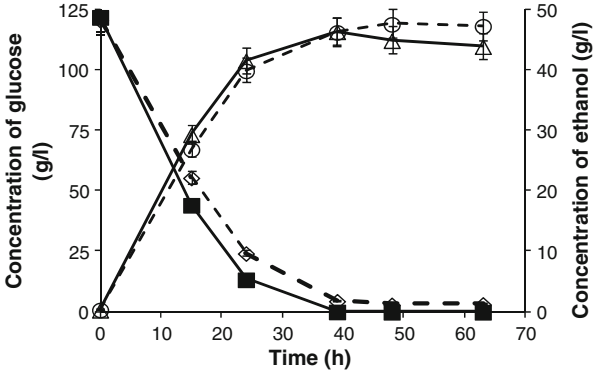

Fig. 1 Fermentation of glucose in the synthetic medium by *Candida shehatae* ATCC 22984 or the mutant Cs3512: (*squares*) glucose of ATCC 22984, (*diamonds*) glucose of Cs3512, (*triangles*) ethanol of ATCC 22984, and (*circles*) ethanol of Cs3512

benefited from that Cs3512 produced 59% less glycerol, which is the major by-products of glucose fermentation [22] and 11% less biomass (data not shown) than ATCC 22984 strain. The production of glycerol is primarily linked to the redox metabolism that is caused by the yeast biomass synthesis from glucose and ammonia [23]. Therefore, the reduction of glycerol production by Cs3512 might have partially resulted from the decrease of biomass production.

Cs3512 displayed significant improvement in xylose fermentation (Fig. 2), which was different from the fermentation of glucose. Finally, 44.4 g/l ethanol was produced by Cs3512 in 48 h, which was 13% higher than that by ATCC 22984. The improvement in xylose fermentation might mainly be attributed to the reduction of xylitol production (Fig. 2) and biomass production (data not shown). ATCC 22984 produced xylitol at the yield of 0.11 g xylitol per gram xylose, while Cs3512 produced xylitol only at the yield of 0.08 g xylitol per gram xylose. Xylitol is the main by-products of xylose fermentation. Its accumulation is partially resulted from the unbalance of NADH and NAD^+ at oxygen-deficient environment [22]. Most of the xylose-fermenting yeasts produce small amount of xylitol even at well-

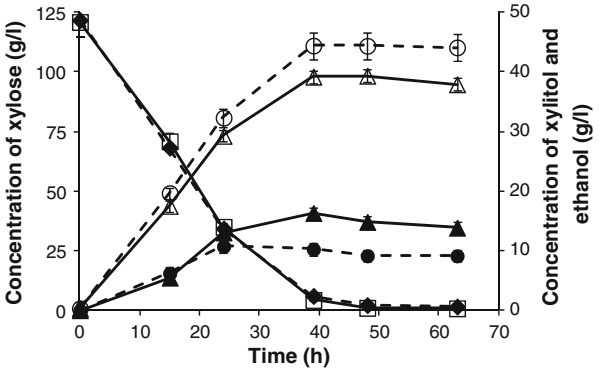

Fig. 2 Fermentation of xylose in the synthetic medium by *Candida shehatae* ATCC 22984 or the mutant Cs3512: (*squares*) xylose of ATCC 22984, (*diamonds*) xylose of Cs3512, (*open triangles*) ethanol of ATCC 22984, (*open circles*) ethanol of Cs3512, (*closed triangles*) xylitol of ATCC 22984, and (*closed circles*) xylitol of Cs3512

controlled aeration conditions [24]. The results here indicated that by selecting the mutants with TTC, we could relieve the unbalance between NADH and NAD^+ to some extent to reduce the production of xylitol and further to improve the ethanol yield.

Antimycin A is an inhibitor of cytochrome c reductase [25]. The colonies that could grow on antimycin A agar plate would have some changes in the structure/function of cytochrome c reductase. Meanwhile, TTC is an indicative chemical which could be reduced by the mitochondrial dehydrogenases to a red reductive product formazan [19]. It was reported that formazan was only accumulated under strictly anaerobic conditions [26]. The strictly anaerobic condition in mitochondria was speculated to be maintained by the cytochrome c which has a very high affinity for oxygen [27]. Therefore, Cs3512 whose color changed to dark red might have relatively high affinity of cytochrome c to oxygen or high activity of dehydrogenase which related to the oxidation of NADH. Therefore, Cs3512 might have the potential to recycle NADH more efficiently at lower oxygen concentration than the other strain that showed light red color. For xylose fermentation yeast *C. shehatae*, xylose was reduced to xylitol by NADPH, and xylitol was oxidized to xylulose by NAD^+. It was speculated that the difference between utilization of the co-factors of NADPH and NAD^+ resulted in the accumulation of xylitol, which would result in the decrease of ethanol production [28]. Hence, high efficiency of NADH recirculation would improve ethanol production by *C. shehatae*. The corresponding reduction of xylitol accumulation in fermentation of xylose by the mutant was well agreed with this presumption (Fig. 2).

In the next step, the capability of Cs3512 to ferment the mixtures of glucose and xylose was studied. One of the media contained 52.9 g/l glucose and 21.2 g/l xylose (called medium A), and the other contained 81.8 g/l glucose and 19.5 g/l xylose (called medium B). The results were listed in Table 1. Both ATCC 22984 and Cs3512 consumed the sugars efficiently. Nearly all of the sugars (97%) were fermented within 40 h. Finally, Cs3512 showed 8% increase of ethanol production to 31.0 g/l from 28.7 g/l by ATCC 22984, 37% reduction of xylitol to 1.81 g/l from 2.86 g/l by ATCC 22984, and 42% reduction of glycerol production to 1.26 g/l from 2.16 g/l by ATCC 22984 in fermentation of sugars in synthetic medium A. In fermentation of sugars in medium B, Cs3512 produced 7% more ethanol (41.1 g/l) than ATCC 22984 (38.5 g/l), 44% less xylitol (2.03 g/l) than ATCC 22984 (3.59 g/l), and 48% less glycerol (1.72 g/l) than ATCC 22984 (3.28 g/l). *C. shehatae* was reported having the diauxic behavior in the fermentation of sugar mixtures that the presence of glucose would inhibit the fermentation of xylose [29]. Although there was little improvement in the fermentation of xylose until the exhaust of glucose, the overall

Table 1 Fermentation of glucose and xylose in synthetic media

	YPM medium A[a]		YPM medium B[b]	
	ATCC 22984	Cs3512	ATCC 22984	Cs3512
Sugar utilization	99%	98%	99%	97%
Ethanol production (g/l)	28.7	31.0	38.5	41.1
Ethanol yield (g ethanol/g sugar consumed)	0.39	0.43	0.39	0.42
Xylitol (g/l)	2.86	1.81	3.59	2.03
Glycerol (g/l)	2.16	1.26	3.28	1.72
Xylitol yield (g xylitol/g xylose consumed)	0.14	0.09	0.20	0.13

[a] YPM medium A was composed of YPM, glucose (52.9 g/l), and xylose (21.2 g/l)

[b] YPM medium B was composed of YPM, glucose (81.8 g/l), and xylose (19.5 g/l)

ethanol production by Cs3512 was significantly improved due to the higher xylose fermentation ability of Cs3512 (Fig. 2).

The xylitol production increased along with the increased ratio of glucose in the mixtures by either ATCC 22984 or Cs3512 (Table 1). The lowest xylitol yield was obtained from the fermentation of 121.3 g/l of xylose in the xylose-only medium which was 0.11 g/g for ATCC2294 and 0.08 g/g for Cs3512. One reason of the accumulation of xylitol in the presence of glucose might be the inhibitory effect of ethanol on the fermentation activity and growth of *C. shehatae*. After the fermentation of 50 g/l or higher concentrations of glucose, the ethanol concentration would exceed 20 g/l before the starting of xylose fermentation [30]. Because the growth and fermentation activity of *C. shehatae* would decrease when the ethanol concentration exceeded 20 g/l [31], the presence of high concentration of glucose would attribute to the deterioration of xylose fermentation. As a result, with the increase of glucose concentration, more xylitol accumulated. Although the influence of glucose on xylose fermentation (diauxic behavior) was not amended by the mutation, the mutants screened by TTC still showed improvement in the final ethanol production from the sugar mixtures with concomitant of improved xylose fermentation.

Fermentation of Rice Straw via SSF

The capability of mutant Cs3512 to ferment the CaCCO-processed rice straw was further researched. The newly developed CaCCO process is able to significantly improve the hydrolysis of biomass and simplify the fermentation process by reducing the separation and clean process [18]. The results were showed in Fig. 3. The sugars, which were hydrolyzed from the rice straw and were mainly composed of glucose and xylose, were utilized by Cs3512 to undetectable value after 72 h of cultivation. Finally, 21.3 g/l of ethanol was produced from the pretreated rice straw (10% of solid content (*w*/*w*)). The resulted overall ethanol conversion efficiency was equivalent to 77% of the theoretical yield based on the total amount of glucan and xylan in the native rice straw. The saccharification of CaCCO-processed rice straw (cv. *Koshihikari*) carried out by Park et al. [18] indicated that the saccharification efficiency of glucose and xylose from the glucan and xylan in the rice straw were 85% and 70%, respectively (cultivation at 50 °C for 24 h). Because it is difficult to know the actual saccharification efficiency in SSF process, here we simply use the saccharification efficiency above to calculate how much sugars were released during SSF

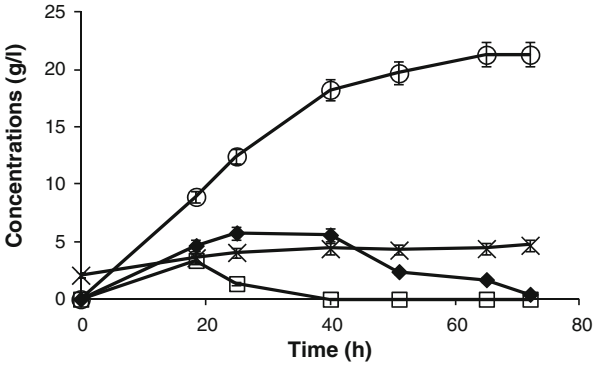

Fig. 3 Fermentation of the lime-pretreated rice straw via SSF by the mutant Cs3512: (*diamonds*) xylose, (*circles*) ethanol, (*squares*) glucose, and ("x") glycerol. *Asterisks* indicate the peak of xylitol was covered by the impurity in the hydrolysate and could not be properly analyzed

and the ethanol yield from these sugars. According to this assumption, the ethanol conversion efficiency reached 96% of the theoretical yields based on the released glucose and xylose. Although there would be some errors because the actual saccharification efficiency might be higher in SSF process [32], as a reference, it implied that Cs3512 could efficiently ferment the glucose and xylose that was hydrolyzed from CaCCO-processed rice straw.

Karimi et al. [33] previously reported on the SSF of dilute-acid pretreated rice straw by *Mucor indicus*, *Rhizopus oryzae*, and *Saccharomyces cerevisiae*. The best ethanol conversion efficiency based on the available glucan was 74% of the theoretical yield obtained by *R. oryzae*. Bertilsson et al. [34] researched the SSF of SO_2-impregnated and steam-pretreated spruce by the recombinant *S. cerevisiae* TMB 3400. They reported that in the batch fermentation of 10% WIS (water insoluble solid), the overall ethanol conversion efficiency based on the fermentable sugars was 75% of the theoretical yield, and after pre-fermentation of the glucose in the hydrolysate before SSF, the ethanol yield increased to 80% of the theoretical yield. Although it is hard to directly compare the results between different researches of producing ethanol from lignocellulosic biomass via SSF because of the different pretreatment processes and fermentation parameters applied, it is still useful to show that Cs3512 compares favorably with some other yeasts and is potentially valuable to ferment lignocellulosic biomass [4, 35–37].

Conclusion

A mutant Cs3512 was obtained from *C. shehatae* ATCC 22984 which produced more ethanol and less xylitol from the sugar mixtures of glucose and xylose. It also showed potential in SSF of lime-pretreated rice straw with high ethanol yield. The results indicate that Cs3512 is a useful non-recombinant stain for ethanol production from lignocellulosic biomass. In the future, further breeding will conduct to reduce the inhibition of glucose on xylose fermentation and improve the ethanol tolerance of Cs3512.

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