

Xylitol Production from Non-detoxified Napiergrass Hydrolysate Using a Recombinant Flocculating Yeast Strain

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Xylose derived from lignocellulose can be utilized to produce ethanol and other high-value chemicals, such as xylitol. The xylitol production through fermentation of lignocellulosic hydrolysate by microorganisms offers advantages of high product yield, high selectivity, and efficacy in mild conditions. In this study, non-detoxified hemicellulose hydrolysate from napiergrass was used for xylitol production by a recombinant flocculating strain of *Saccharomyces cerevisiae*. An optimization study was conducted with the strain at 35 °C. A promising xylitol yield of 0.96 g/g xylose with no addition of glucose required during the fermentation process, which suggests an extensive potential improvement for the economics of lignocellulosic xylitol production.

Keywords: *Saccharomyces cerevisiae*; *Hemicellulosic hydrolysate*; *Xylitol*; *Recombinant yeast*; *Xylose utilization*

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INTRODUCTION

Xylitol, a sugar substitute, has sweetness equivalent to that of sucrose, but with a lower caloric content. It is attracting global attention because of its various properties, such as prevention of dental caries and insulin independence in human metabolic regulation. Therefore, it is widely used as a sucrose substitute in the diets of individuals with clinical diabetes. Industrially produced xylitol has applications in at least three major manufacturing processes, namely, the food, chemical, and pharmaceutical industries. Currently, xylitol is technically produced from hemicellulosic hydrolysate containing xylose, and production involves a costly and energy-intensive chemical process, which converts xylose to xylitol through hydrogenation with an expensive metal catalyst under extreme conditions (Rao *et al.* 2016). In addition, traditional chemical conversion requires a complex separation and purification process to remove by-products, and the yield of xylitol produced by this chemical synthesis can be as low as 50% to 60% (Rafiqul and Sakinah 2013). To reduce production cost and meet the increasing market demand for xylitol, research efforts have focused on alternative biological production methods that employ yeast for fermentation using cheap lignocellulosic materials, such as agricultural waste and forest residues.

Lignocellulosic materials are plentiful, renewable, and inexpensive sources of sugars and comprised mainly of cellulose, hemicellulose, and lignin. Hemicellulose content is 20% to 35%, and it can be converted into xylose through saccharification methods, including pretreatment and enzymatic hydrolysis. Dilute sulfuric acid hydrolysis and steam explosion is a common pretreatment method to release fermentable sugars,

especially for converting hemicellulose to pentose (Chen *et al.* 2011). This method has been applied to various lignocellulosic biomasses, such as wood, sugarcane bagasse, corncob, and rice straw, for xylitol production. During acid pretreatment, various inhibitors, mainly furfural and 5-hydroxymethylfurfural (5-HMF), are generated and negatively affect cell growth and the xylitol fermentation process (Mussatto and Roberto 2004). Additionally, the residual xylan in the hydrolysate prohibits strains from completely fermenting xylose; therefore, extra glucose must be added during the xylose-conversion process to achieve a high xylitol yield (Silva *et al.* 2007). However, this glucose feeding results in complex production procedures and increased cost. Consequently, lignocellulosic hydrolysate detoxification and the use of inhibitor-tolerant microorganisms without additional glucose feed have been used to counteract this inhibition problem. Detoxification strategies have limitations, including sugar loss and additional filtration steps, which reduce fermentation efficiency (Villarreal *et al.* 2006). Another alternative to circumvent the inhibitor problems of xylitol production from lignocellulosic hydrolysates is to enhance the inhibitor tolerance of fermenting microorganism.

In a previous study (Ma *et al.* 2012), a genetically engineered a strain of xylose-utilizing *Saccharomyces cerevisiae* was developed with improved capacity for converting xylose into ethanol. Numerous efforts have focused on the initial xylose metabolic pathway in *S. cerevisiae* for its critical role in xylose utilization. Several reports have demonstrated xylitol-producing *S. cerevisiae* that acquired tolerance toward HMF and furfural because of the expression of xylose reductase obtained from *Candida* species in *S. cerevisiae* (Heer *et al.* 2009). However, flocculating yeast strains perform well in fermenting inhibitory hydrolysates (Westman *et al.* 2012), and flocculation has also been reported to increase the inhibitor tolerance of *S. cerevisiae* (Martín and Jönsson 2003; Purwadi *et al.* 2007). Another important advantage of using flocculating yeast is that it is extremely fast to flocculate. This property can potentially be used to separate yeasts easily at the end of the fermenting process and may be used for an economical and cost-competitive method of xylitol production using lignocellulosic material.

The study analyzed the fermentation characteristics of a genetically engineered flocculating *S. cerevisiae* strain, named 550C1, containing the xylose reductase gene from *Candida guilliermondii* for producing xylitol from non-detoxified napiergrass hydrolysate. The results of this study may provide an effective xylitol production method, which can potentially be applied in xylitol production.

EXPERIMENTAL

Strains and Plasmids

S. cerevisiae strain YYA1 (ADH2::PGKIp-CXYL1-ADHlt, XKS1p::PGKp, HO::PGKI_p-XYL2-ADHlt, Mig1::TEFp-TAL1-TEFt) was used for cloning of the xylose reductase gene (XYL1) from *C. guilliermondii* (hereafter “CXYL1”) (Ma *et al.* 2012). The flocculent strain WLP 550 was purchased from White Labs (San Diego, CA) and used as a host for xylitol production. In the present study, the plasmid pAURC1 was prepared in accordance with the construction method of Ma *et al.* (2012). Briefly, plasmid pAURC1 containing the CXYL1 gene under the phosphoglycerate kinase (PGK) promoter was constructed on the basis of the pAUR101 shuttle vector (Takara Bio, Kyoto, Japan). The sequence of PGK-CXYL1 was amplified by PCR using two primers (GGTACCGA-GGAGCTTGGAAAGATGCC and GGTACCCTGGAGCTCATGCTATACCTGAG)

with engineered *KpnI* and *SacI* sites of primers. The amplified CXYL1 gene was cloned into the pAUR101 vector including Aureobasidin A (AbA)-resistance gene (AUR1-C). The resulting plasmid was designated pAURC1 (Fig. 1).

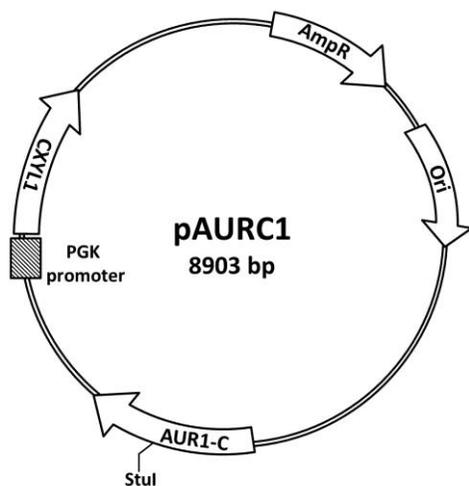


Fig. 1. Genetic map of plasmid pAURC1

Transformation

Yeast transformation of the plasmid pAURC1 was performed through electroporation. For electroporation, pAURC1 was linearized with *StuI*, and the linearized vectors were transformed into *S. cerevisiae* WLP 550 using the lithium acetate method and then conducted using a Bio-Rad Gene Pulser Xcell (Richmond, CA, USA) with the electric shock at 1,500 volts and 25 microfarads (Ma *et al.* 2012). Following electroporation, the obtained transformants that express the gene encoding CXYL1, named 550C1, were immediately plated on selective yeast-extract peptone dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% glucose) containing 0.5 mg/L of aureobasidin A (Takara Bio, Kyoto, Japan). Transformants appeared within 3 days at 30 °C. Well-grown colonies with different xylose fermentation capacities were selected.

Media and Culture Conditions

Saccharomyces cerevisiae strains and transformants were grown in YPD medium. Cultivation of *S. cerevisiae* was performed at 30 °C and 150 rpm.

Microorganism and Inoculum Preparation

Saccharomyces cerevisiae 550C1 was used for the bioconversion of the xylose present in hydrolysate into xylitol. Inoculation cultures were grown in YPD medium and incubated at 30 °C in a shaker with a rotation speed of 200 rpm for at least 24 h prior to harvesting by centrifugation. An initial dry cell weight (DCW) was 1.5 g/L for both bench- and pilot-scale bioreactors.

Xylitol Fermentation Studies

Bench- and pilot-scale xylitol production was performed in bioreactors with total volumes of 5 L and 1000 L using fermentative media simulating hemicellulose hydrolysate sugar compositions (containing 1% yeast extract, 2% peptone, 4% xylose supplemented with 0.5% glucose, and inoculum material) corresponding to 2 L and 400 L, respectively.

The fermentation temperature used was 30 °C, and agitation was performed at 200 rpm for 48 h. Samples were taken periodically for HPLC analysis.

Preparation of Hemicellulosic Hydrolysate of Napiergrass

Napiergrass is an abundant lignocellulosic waste in Taiwan, and it has high glucan (36% to 40%) and xylan (21% to 23%) content (Mohammed *et al.* 2015; Tsai *et al.* 2018). In this study, napiergrass was collected from a field near Taoyuan in Taiwan. The feedstock was air-dried and sliced to less than 2 cm. The raw material was stored at room temperature until further processing. The overall procedures of pretreatment include acid-soaking, solid-liquid separation, and steam explosion. Briefly, 3 kg (based on dry weight) feedstock was firstly soaking in 20 kg sulfuric acid solution with concentration from 1% to 1.5%. After that the feedstock slurry above was centrifuged to remove excess acid liquid and the solid part was retained. The impregnated feedstocks containing acid above were then introduced into a 20-L vessel designed as a steam explosion reactor (Lucky Seven Industrial Co., Ltd., Taoyuan, Taiwan). Saturated steam was allowed to enter the reactor and heated the raw material to 180 °C with different reaction time (1, 5, 10 min), and then the reactor was suddenly depressurized. A flash tank with a cooling system was used to collect the exploded material as the mixture was cooled, and the hydrolysate with volume about 4~7 L was obtained through slurry filtration, in which acid-treated slurry was immediately filtered to separate the solid and liquid fractions. The liquid fraction was the hydrolysate used as the fermentation medium (Guo *et al.* 2009).

Napiergrass Hemicellulosic Hydrolysate Fermentation

Xylitol production from napiergrass hemicellulosic hydrolysate was performed in a 1-L bioreactor. Before the fermentation was performed with the yeast, the pH of the hydrolysate was adjusted to 6.0 using 10 N NaOH. The hemicellulosic hydrolysate was supplemented with 0.5% yeast extract as nitrogen sources. Then *S. cerevisiae* 550C1 was inoculated into 0.3 L of the fermentation medium, and fermentation was performed at 30 °C with an agitation speed of 200 rpm for at least 48 h.

Analytical Methods

All liquid samples were filtered through a 0.22- μ m filter and diluted appropriately with deionized water. Quantitative analysis of glucose, xylose, xylitol, and furfural was performed on an Agilent 1200 HPLC system (Santa Clara, CA, USA) equipped with a refractive index detector. Separation was performed using a Coregel-87H3 column (Transgenomic, San Jose, CA, USA) at 65 °C with 4 mM H₂SO₄ as eluent and at a flow rate of 0.5 mL/min.

RESULTS AND DISCUSSION

In a previous study (Ma *et al.* 2012), the authors found that overexpression of the *C. guilliermondii* XYL1 under the transcriptional control of the PGK promoter increased the xylitol productivity of *S. cerevisiae*. In present study, the focus was on genetically modifying the flocculating yeast strain *S. cerevisiae* 550 for the production of xylitol. Through chromosomal integration as previously described (Ma *et al.* 2012), the *S. cerevisiae* 550 was genetically modified with XYL1 from *C. guilliermondii*, resulting in *S. cerevisiae* 550C1, and this was used for xylose fermentation study.

Batch fermentations for xylitol production on bench scale and pilot scale were performed with *S. cerevisiae* 550C1 grown in the fermentative medium simulating hemicellulose hydrolysate sugar composition and supplemented with peptone and yeast extract to predict the strain's behavior in hydrolysate. Figure 2 displays the time course of xylose consumption and xylitol production by *S. cerevisiae* 550C1 in the simulated hydrolysate medium in the 5 L (Fig. 2a) and 1000 L (Fig. 2b) bioreactors. After 24 h of fermentation, the xylitol yield was 0.87 g/g and 0.91 g/g, almost similar to theoretical yields (Barbosa *et al.* 1988), when the 5 L and 1000 L bioreactors, respectively, were used. As summarized in Fig. 2c, the fermentative behavior was similar and independent of the reactor volume. These results and observations indicates that *S. cerevisiae* 550C1 is effective for xylitol production and that the process is scalable.

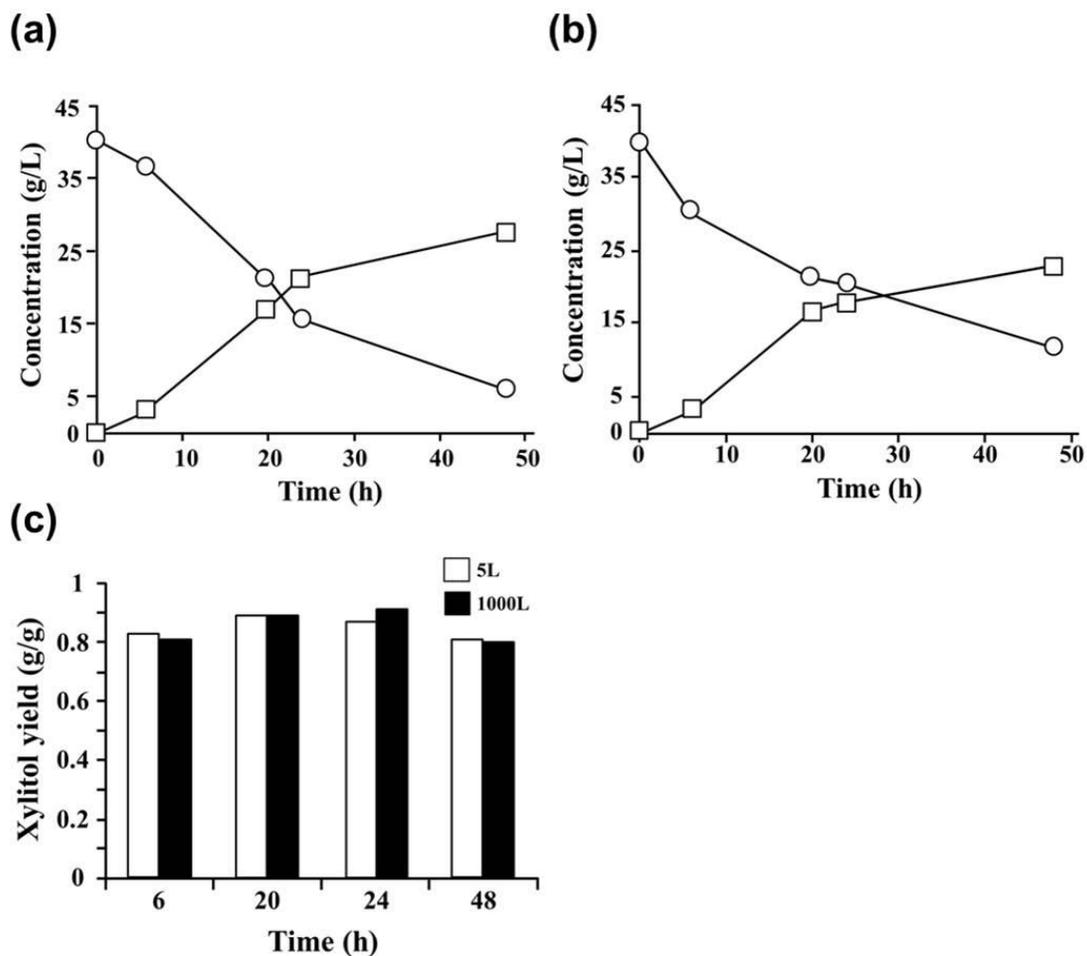


Fig. 2. Xylitol fermentation by *S. cerevisiae* 550C1 from xylose in a 5 L (a); and 1000 L (b) bioreactors. Xylitol fermentation using 40 g/L xylose as a substrate at 30 °C: Square: xylitol; and circle: xylose. (c): Xylitol yield obtained at bench (5 L) and pilot scales (1000 L)

A crucial step in xylitol production is the pretreatment process, which is essential for removal of lignin and destruction of the biomass crystallinity. A commonly used technique among the various types of pretreatments is dilute acid hydrolysis of lignocellulosic biomass followed by steam explosion, and during this process, various parameters must be optimized for xylose recovery. Thus, it is essential to evaluate the napiergrass hydrolysate prepared using various severity conditions of pretreatment.

Table 1 lists the constituents of the six hydrolysates, labeled as P0–P5, which were produced as described. The acid-catalyzed steam explosion process was conducted at 180 °C, with a reaction time of 1.0 to 10 min and at initial H₂SO₄ concentrations of 1% to 1.5%.

Table 1. Constituents of Nondetoxified Napiergrass Hydrolysates after Dilute Acid Pretreatment at Various Severities

Hydrolysate sample	P0	P1	P2	P3	P4	P5
Temperature (°C)	180					
Time (min)	1	5	10	1	5	10
H ₂ SO ₄ (%)	1	1	1	1.5	1.5	1.5
Glucose (g/L)	1.0	1.95	3.24	2.87	4.9	3.6
Xylose (g/L)	20.7	21.3	22.6	28.5	35.3	12.1
Furfural (g/L)	0.58	1.0	0.14	1.02	0.72	2.98

After pretreatment, the napiergrass hydrolysates were enriched with xylose- and inhibitor media. Comparison of the components of the hydrolysates revealed that those pretreated with 1.5% H₂SO₄ (P3, and P5) released more furfural. Batch fermentation of the non-detoxified napiergrass hydrolysate at 1.5 g/L dry cell weight starting cell density was conducted, and the results are presented in Fig. 3. After 24 h of fermentation, the similar xylitol yield was obtained from fermentation of the hydrolysate under various severities, and the P5 hydrolysate had the lowest xylose consumption rate due to a higher concentration of furfural. According to xylitol yield and the xylose consumption rate obtained in the presence of furfural without additional glucose adding, *S. cerevisiae* 550C1 could produce xylitol from the fermentable sugars in the non-detoxified hemicellulosic hydrolysates. This might be attributed to the higher initial cell concentration, since this work already increased the cell mass in the fermentation broth, suggesting without the need of extra glucose to grow cells. Further, the data from this study may mean the flocculating property of the 550C1 strain that keeps high cell density probably gives a possibility for inner lying yeasts to have chances to survive and work in toxic hydrolysates, likely through the protection of the outer layer cells.

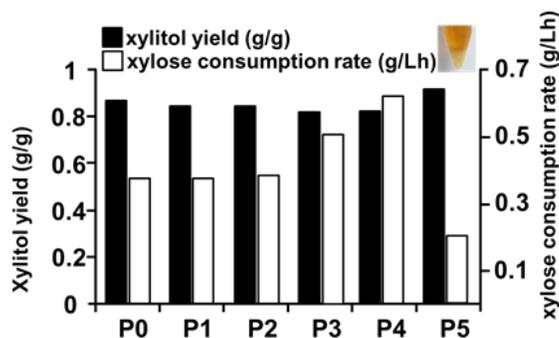


Fig. 3. Results for *S. cerevisiae* 550C1 fermentation of non-deoxified napiergrass hydrolysate (Inset: Flocculating stereomicrograph image of *S. cerevisiae* 550C1 after resuspension in medium)

To improve the bioconversion of hydrolysates into xylitol using the 550C1 strain, optimization of the fermentative parameters, such as temperature, pH, and nitrogen source (urea) concentration were evaluated on the basis of the xylose recovered from the

napierrgrass hydrolysate. Unless otherwise stated, the temperature was 30 °C, initial pH was 6.0, and yeast extract content was 0.5%. The effects of these parameters on xylitol yield, xylose consumption rate, and xylitol productivity were investigated. As shown in Fig. 4, fermentation was almost independent of pH and urea content when the yeast was kept in media at pH 5 to 7, and 1% to 3% urea concentration ranges. However, the results demonstrated that temperature had significant effects on xylitol yield and xylose consumption rate in temperature range 30 °C to 39 °C. The maximal xylitol yield obtained was 0.96 g/g at 35 °C, and the maximal xylitol productivity was 0.44 g/Lh achieved at 30 °C.

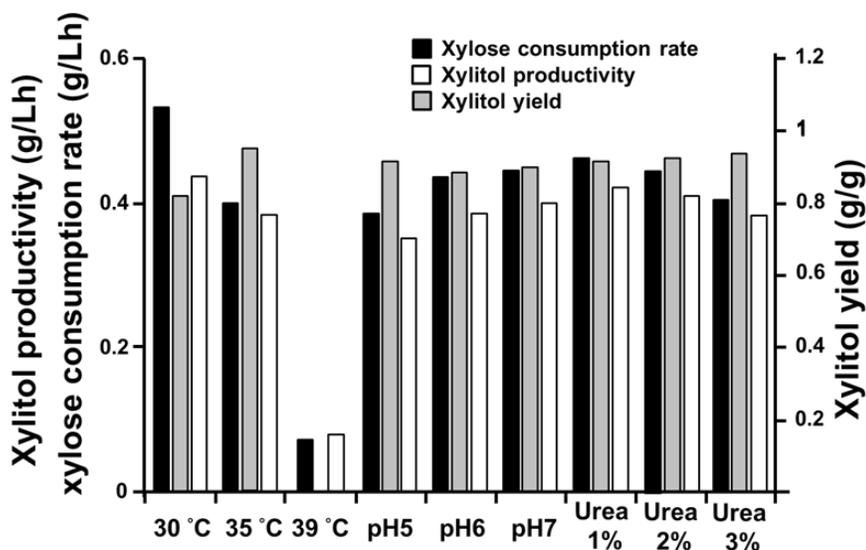


Fig. 4. Fermentative parameters for xylitol production by non-detoxified *S. cerevisiae* 550C1 subjected to various conditions.

Several strains of *S. cerevisiae* were used to ferment hemicellulose hydrolysate from various lignocellulosic materials for xylitol production, and Table 2 compares the fermentation results. The maximal xylitol yield from these materials differed widely from 0 to 1 g/g, depending on the media composition and xylose-metabolism-related enzyme activity. However, the 550C1 *S. cerevisiae* demonstrated great potential for xylitol production with a yield of 0.96 g/g from non-detoxified hydrolysate. This was as high as or higher than yields that have been reported in other published studies.

Table 2. Comparison of Xylitol Production by *S. cerevisiae* among Various Studies

<i>S. cerevisiae</i> Strain	Feedstocks	Initial Xylose (g/L)	Xylitol Yield (g/g)	Refs.
<i>S. cerevisiae</i> PE-2	corn cob hydrolysate	27.2	0.89	(Baptista <i>et al.</i> 2018)
<i>S. cerevisiae</i> CEN.PK-pXks	wood hydrolysate	39	0.2	(Johansson <i>et al.</i> 2001)
<i>S. cerevisiae</i> SCX-5	xylose	20	1	(Zha <i>et al.</i> 2013)
<i>S. cerevisiae</i> YY2KL	xylose	20	0.1	(Ma <i>et al.</i> 2012)
<i>S. cerevisiae</i> 550C1	napierrgrass hydrolysate	35.3	0.96	This work

CONCLUSIONS

1. The potential of a new recombinant flocculating strain of 550C1 *S. cerevisiae*, which is transformed with the xylose reductase gene (XYL1), to provide a high xylitol yield of 0.96 g/g xylose when used with nondetoxified acid-pretreated napiergrass hydrolysate was studied. No additional glucose was required during the fermentation process; thus, fermentation in this manner can prevent contamination of the hydrolysate-based medium. Moreover, the flocculation characteristics of this strain not only provide the necessary inhibitor tolerance but also enable easier recovery of the yeast and thus increase the economic advantages of this strain for xylitol production.
2. Fermentation was successfully scaled up from the bench to pilot scale, and the pilot-scale xylitol yield data indicated that the 550C1 strain of *S. cerevisiae* can potentially be exploited for industrial bioproduction.

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