Ethanol Production from Sugarcane Leaves by *Kluyveromyces marxianus* S1.17, a Genome-Shuffling Mediated Transformant

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Kluyveromyces marxianus S1.17, obtained by electroporation-mediated genome shuffling between *K. marxianus* G2-16-1, a cellobiase-producing yeast, and *Pichia stipitis* JCM 10742^T, gave a maximum ethanol production level (of 0.86 g/L) from the hydrolysate of dilute sulfuric acid treated sugarcane leaves when treated under aerobic conditions for 72 h, compared to the ethanol production level of 4.73 g/L from the acid-treated sugarcane leaves fermented by the simultaneous saccharification and fermentation process under oxygen-limited conditions. The total ethanol produced from sugarcane leaves by *K. marxianus* S1.17 was 5.59 g/L (0.10 g/g, dry weight).

Keywords: Kluyveromyces marxianus; Ethanol; Sugarcane leaves; Genome modified

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INTRODUCTION

Thailand is the fourth largest cane sugar exporter in the world. However, 65 million tons of sugarcane is cultivated in Thailand annually (Ngugen *et al*. 2010), and up to 40% of the harvested sugarcane biomass is unused leaves (Moodley and Gueguim Kana 2015). This is because the leaves have a rough texture and sharp edges that make them unsuitable for animal feed and so become an agricultural waste product. Moreover, the sharp leaves make cane harvesting difficult, and so most farmers burn the leaves before harvesting, which causes localized air pollution and wastes their inherent energy value. Indeed, because of the high cellulose (39 to 40%, w/v) and hemicellulose (23 to 27%, w/v) contents of sugarcane leaves (Krishnan *et al*. 2010; Moodley and Gueguim Kana 2015), they are of interest as a potential raw material for the production of ethanol and other high-value products. Recently, it was found that the amount of xylose released by the dilute sulfuric acid treatment of sugarcane leaves was almost the same as the amount of glucose released by the subsequent enzymatic hydrolysis of the acid-treated sugarcane leaves (Jutakanoke *et al*. 2012). Therefore, in order to maximize the ethanol production from sugarcane leaves, the xylose in the acid-treated leaf hydrolysate as well as the glucose from the hydrolysis of the acid-treated sugarcane leaves should be fermented to ethanol.

Kluyveromyces marxianus G2-16-1, a high-glucose ethanolic fermenting yeast that produces cellobiase, was initially isolated from a sugar factory in Thailand. Cellobiase is an enzyme that hydrolyzes cellobiose, the accumulation of which would otherwise inhibit the cellulose hydrolysis to glucose. The optimal temperature for ethanol production by *K. marxianus* G2-16-1 was found to be 40 °C (Akaracharanya *et al.* 2016), making it suitable for use in lignocellulosic ethanol production by the simultaneous saccharification and fermentation (SSF) method, since this is required to be operated at a temperature close to the optimal temperature of cellulase (40 to 50 °C) (Olsson and Hahn-Hägerdal 1996). The SSF method of producing ethanol from lignocellulose uses the simultaneous hydrolysis of cellulose into glucose and the fermentation of the obtained glucose into ethanol. Thus, there is no glucose accumulation that would otherwise inhibit the cellobiose hydrolysis (Wingren *et al.* 2003; Saha *et al.* 2011). This results in a higher total amount of glucose being released from the cellulose and consequentially a higher total ethanol production level. However, *K. marxianus* G2-16-1 has a low efficiency for xylose fermentation.

Genome shuffling is a popular method of improving industrial microbial strains, because it is rapid, convenient, and has a high potential to obtain the desired phenotypic strain (Liu *et al.* 2006). To improve the ability of *K. marxianus* to ferment xylose into ethanol, *K. marxianus* G2-16-1 was subjected to electroporation-mediated genome shuffling (Zhang and Geng 2012) using the chromosomal DNA of *Pichia stipitis* JCM 10742^T, a high-xylose fermenting yeast (Du Preez and Prior 1985) as the chromosomal donor. This study is the first report of genome shuffling between *K. marxianus* and *P. stipitis.* Genome-shuffling mediated *K. marxianus* G2-16-1 transformants with an increased xylose fermentation capability were then selected for indirectly by selecting for a high ethanol tolerance.

EXPERIMENTAL

Materials

Sugarcane leaves

Sugarcane (*Saccharum officinarum*) leaves were collected from the Center for Sugarcane and Sugar Industry Promotion (Region 3), Nakhon Ratchasima Province, Thailand. The moisture content of the sugarcane leaves was 86.28% (w/w) dry weight. They were cut, oven-dried (60 °C, 3 days), then hammer-milled to a 20- to 40-mesh particle size. Their cellulose and hemicellulose contents were analyzed using the TAPPI T203 cm-99 (1999), and the lignin content was analyzed using the TAPPI T222 om-02 (2002).

Microorganisms

Kluyveromyces marxianus G2-16-1, a cellobiase-producing yeast, which gave an ethanol yield of 0.36 g/g from a 15% (w/v) glucose-containing medium (pH 5.0) at 40 °C after 48 h (Akaracharanya *et al*. 2016), was used in this study. *Pichia stipitis* JCM 10742^T was obtained from the Japan Collection of Microorganisms, RIKEN BioResource Center, Tsukuba, Japan.

Enzyme

The cellulase used in this study was Accellerase™ 1500 (Genecor International Inc., USA) with an endoglucanase activity of 2,500 CMC (carboxymethyl cellulose) units/g and a β-glucosidase activity of 650 pNPG (p-nitrophenyl glucoside) units/g.

Methods

Ethanol tolerance of K. marxianus G2-16-1

The *K. marxianus* G2-16-1 isolate was cultivated on modified YPD agar (10% (w/v) glucose, 0.3% (w/v) yeast extract, 0.3% (w/v) peptone, and 2% (w/v) agar, pH 4.5) containing various concentrations of ethanol (1 to 6%, w/v) at 40 °C for 7 days. The ethanol tolerance of *K. marxianus* G2-16-1 was defined as the highest concentration of ethanol in the modified YPD agar in which *K. marxianus* G2-16-1 could grow within 7 days.

Strain improvement of K. marxianus G2-16-1 by genome shuffling

The *K. marxianus* G2-16-1 and *P. stipitis* JCM 10742^T isolates were used as the host and donor strains, respectively. The chromosomal DNA of *P. stipitis* JCM 10742^T was extracted following the method of Manitis *et al*. (1982). One loopful of *P. stipitis* JCM 10742^T was suspended in 200 μL lysis buffer (100 mM Tris-HCl, pH 8.0, containing 30 mM EDTA and 0.5% (w/v) SDS), boiled for 15 min, mixed with 200 μL of 2.5 M potassium acetate (pH 7.5), and placed on ice for 1 h. After centrifugation, the supernatant was harvested and extracted twice with an equal volume of chloroform:isoamylalcohol and then precipitated by isopropanol. The DNA pellet was rinsed with 70% (v/v) and 95% (v/v) ethanol, dried at 37 °C, and dissolved in 30 μL of sterile distilled water.

Competent *K. marxianus* G2-16-1 cells were prepared by a modification of the method of Russell *et al.* (1993). Mid-log phase cells were harvested by centrifugation (4 °C, 8000 rpm, 5 min) from the culture (50 mL, OD_{600nm} of 0.6 to 1.0) and washed with an ice-cold electroporation buffer (EB buffer; 10 mM Tris-HCl buffer, pH 7.5, containing 270 mM sucrose and 1 mM $MgCl_2$). The washed cells were re-suspended in 10 mL of YPD broth containing 3% (w/v) ethanol, 0.01% (w/v) chloramphenicol, 25 mM dithiothreitol, and 20 mM Hepes buffer pH 8.0, and then incubated at 30 °C for 30 min. After that, the cells were washed three times with 10 mL EB buffer and re-suspended in 1 mL of EB buffer.

The suspension of competent cells (10 μL) was transformed with the *P. stipitis* JCM 10742^T chromosomal DNA (0.2 μg) by electroporation (Bio-Rad, USA). The transformed cells were suspended in YPD broth (1 mL) and incubated at 40 °C for 3 h before being plated on modified YPD agar containing 4% (w/v) ethanol (selective medium) and incubated at 40 °C for 7 days.

Ethanol production from xylose by K. marxianus G2-16-1

A single colony of *K. marxianus* G2-16-1 grown on YM agar (1% (w/v) glucose, 0.3% (w/v) malt extract, 0.5% (w/v) peptone, 0.3% (w/v) yeast extract, pH 4.5) at 40 °C for 72 h was inoculated into 50 mL of xylose medium (5% (w/v) xylose, 0.3% (w/v) yeast extract, 0.5% (w/v) peptone, pH 5.5) in a 250-mL flask and incubated in a gyrotary shaker (200 rpm) at 40 °C for 48 h. The culture was transferred at 1% (v/v) to a fresh xylose medium and incubated at the same condition for 48 h to become the inoculum.

The inoculum was inoculated at 10% (v/v) into 50 mL of xylose fermentation medium (5.0% (w/v) xylose, 0.3% (w/v) yeast extract, 0.3% (w/v) peptone, pH 5.5) in a 100-mL Duran bottle and incubated at 40 °C on gyrotary shaker for 72 h. Fermentation under aerobic conditions was performed by shaking at 200 rpm, whilst fermentation under oxygen-limited conditions was performed by closing the screw cap of the Duran bottle tightly and subjecting it to slow shaking at 130 rpm. Samples were taken every 24 h, centrifuged (4 °C, 8000 rpm for 10 min), and the supernatant was analyzed for the ethanol level by gas chromatography (GC) as reported (Jutakanoke *et al*. 2012). In comparison, *P. stipitis* JCM 10742^T was used as the control strain and was incubated as above, except at 30 °C.

Pretreatment of sugarcane leaves

Sugarcane leaves were pretreated by dilute sulfuric acid as reported (Jutakanoke *et al*. 2012). In brief, the sugarcane leaves (3 g, dry weight) were suspended in 50 mL of 1.5% (w/v) sulfuric acid and autoclaved at 121 °C, 100 kPa for 30 min. The resulting slurry of acid-treated sugarcane leaves was filtered through stainless steel mesh and centrifuged to separate the acid-treated leaf hydrolysate (supernatant) from the acid-treated sugarcane leaves (residual solid). The glucose and xylose contents in the pretreatment hydrolysate were then analyzed by HPLC as reported (Akaracharanya *et al*. 2011).

Ethanol production from the acid-treated leaf hydrolysate

The acid-treated leaf hydrolysate was adjusted to pH 5.0, supplemented with 0.3% (w/v) yeast extract and 0.3% (w/v) peptone, autoclaved at 110 °C for 10 min, and then used as a fermentation medium. The *K. marxianus* G2-16-1 and *K. marxianus* S1.17 cells, obtained from centrifugation of the inoculum prepared as above, were separately suspended in the fermentation medium at the final cell number of 10^8 cells/mL and incubated under aerobic conditions at 40 °C for 72 h. Samples were taken every 24 h, centrifuged, and the resultant supernatant was analyzed for its ethanol, glucose, and xylose contents by HPLC.

Ethanol production from acid-treated sugarcane leaves

The acid-treated sugarcane leaves were suspended in 100 mM citrate buffer pH 5.0 and hydrolyzed by various doses of Accellerase™ 1500 (364.8 to 2,188.8 CMC units/g, dry weight) at 50 °C (125 rpm) for 6 h, followed by centrifugation at 4 °C (12,000 rpm) for 20 min. The resultant supernatant was analyzed for the glucose content. The minimum dose of Accellerase™ 1500 that released the highest amount of glucose was selected for the hydrolysis of the acid-treated sugarcane leaves thereafter.

The acid-treated sugarcane leaves were then fermented to ethanol by the SSF method. The leaves were first suspended in 50 mL of a 100 mM citrate buffer of pH 5.0 containing 0.3% (w/v) yeast extract and 0.3% (w/v) peptone in a 100-mL Duran bottle, autoclaved at 110 °C for 10 min, and then used as the fermentation medium. Accellerase™ 1500 (1,824 CMC units/g, dry weight) and *K. marxianus* G2-16-1 or *K. marxianus* S1.17 cells (final 10^8 cells/mL) were added. The inoculated fermentation medium was incubated under either an aerobic condition (40 °C, 200 rpm, 72 h) or an oxygen-limited condition (tightly closed screw cap at 40 °C, 100 rpm mixing for 132 h).

Samples were taken every 24 h, centrifuged, and the supernatant was analyzed for the ethanol, xylose, and glucose levels (Fig. 1).

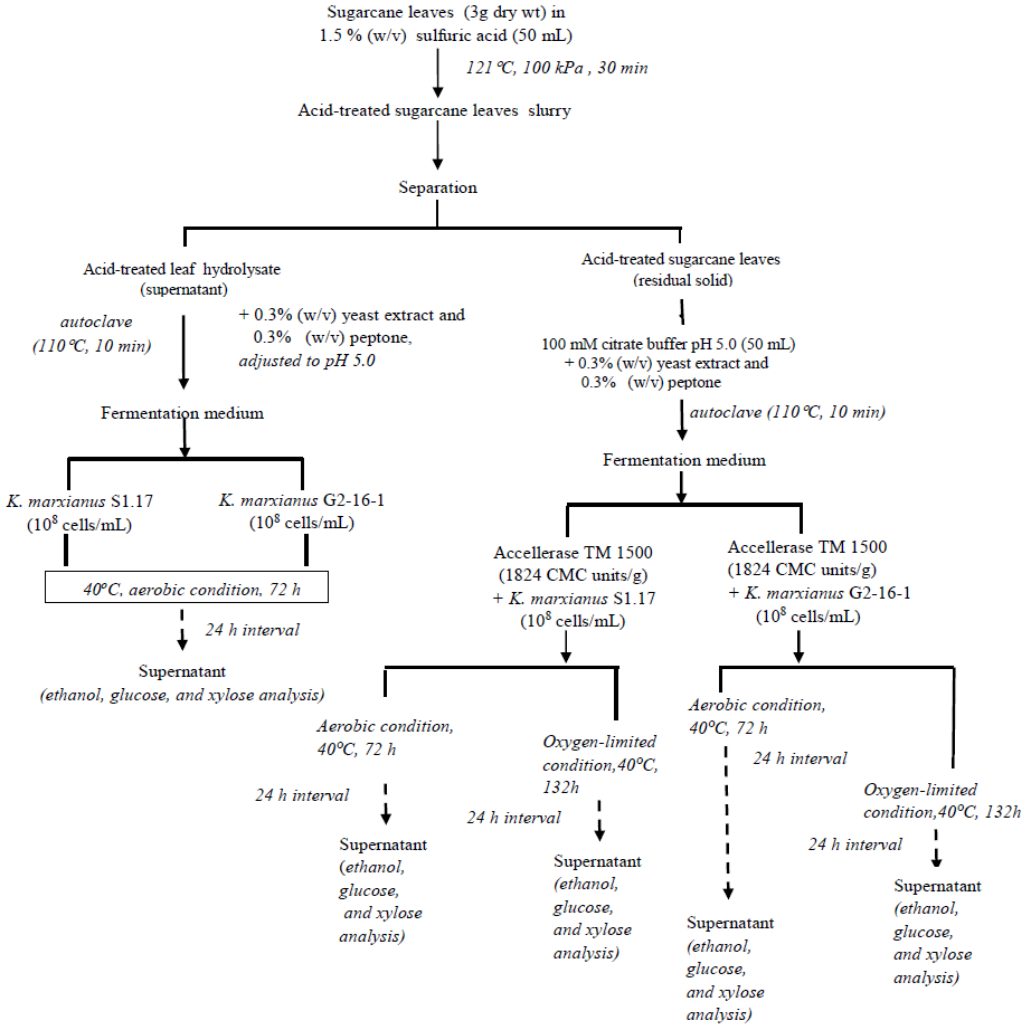


Fig. 1. Schematic summary of the ethanolic fermentation of sugarcane leaves

RESULTS AND DISCUSSION

Sugarcane Leaves

The sugarcane leaves were composed of 39.6% (w/w) cellulose, 27% (w/w) hemicellulose, and 13.9% (w/w) lignin, dry weight.

Ethanol Tolerance of *K. marxianus* G2-16-1

At 40 °C, *K. marxianus* G2-16-1 could grow on modified YPD agar containing 3% (w/v) ethanol after four days of incubation. Therefore, modified YPD agar containing 4% (w/v) ethanol was used as the selective medium to screen for the transformant *K. marxianus* isolates with a suitably modified genome (*i.e.*, greater ethanol tolerance).

Strain Improvement of *K. marxianus* G2-16-1 by Genome Shuffling

After the transformation of *K. marxianus* G2-16-1 with *P. stipitis* JCM 10742^T chromosomal DNA by electroporation-mediated genome shuffling, the cell suspension was spread onto modified YPD agar containing 4% (w/v) ethanol. After incubation at 40 °C for 7 days, 14 transformant colonies were obtained. The analysis of the ethanol production of all the transformants after 48 h in the xylose fermentation medium at 40 °C under oxygen-limited conditions revealed that transformant *K. marxianus* S1.17 isolate produced the highest level of ethanol (0.47 g/L). The subsequent transformation of *K. marxianus* S1.17 by electroporation-mediated genome shuffling using *P. stipitis* JCM 10742^T chromosomal DNA for two more rounds (Fig. 2) yielded 17 and 25 transformant colonies, respectively, but none gave a higher ethanol yield from xylose than *K. marxianus* S1.17.

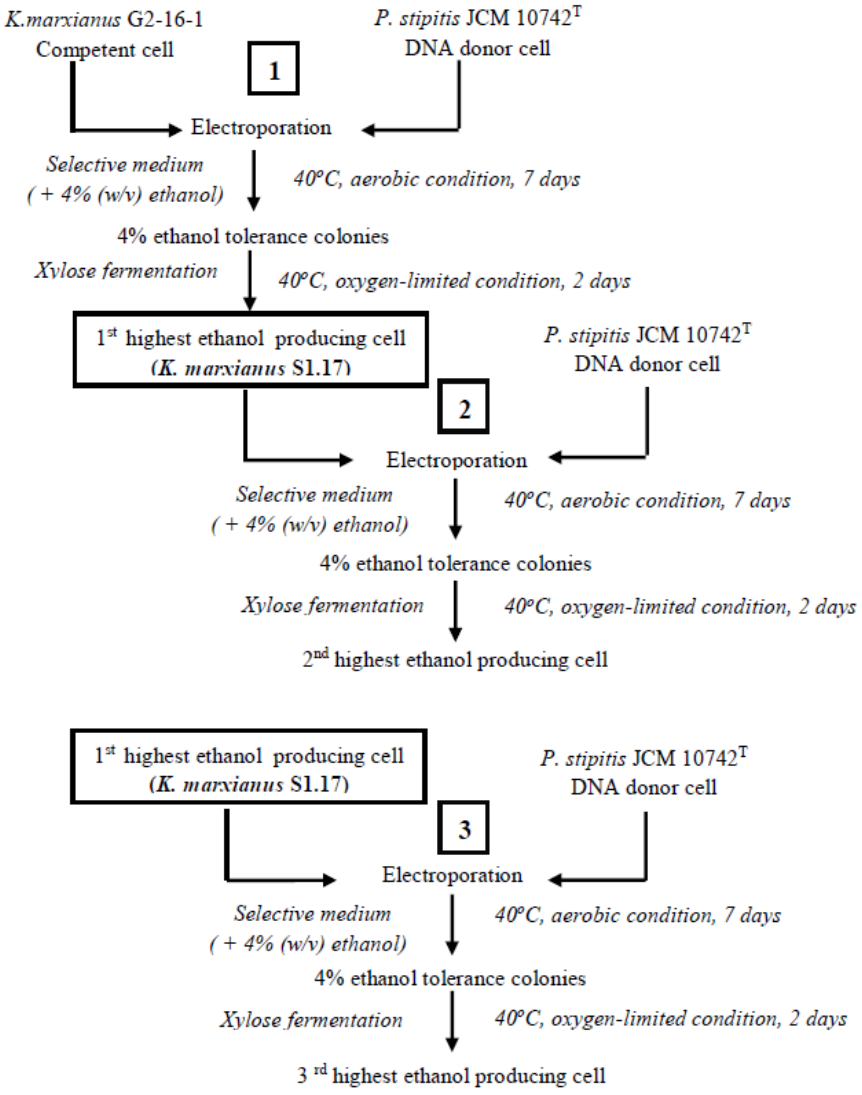
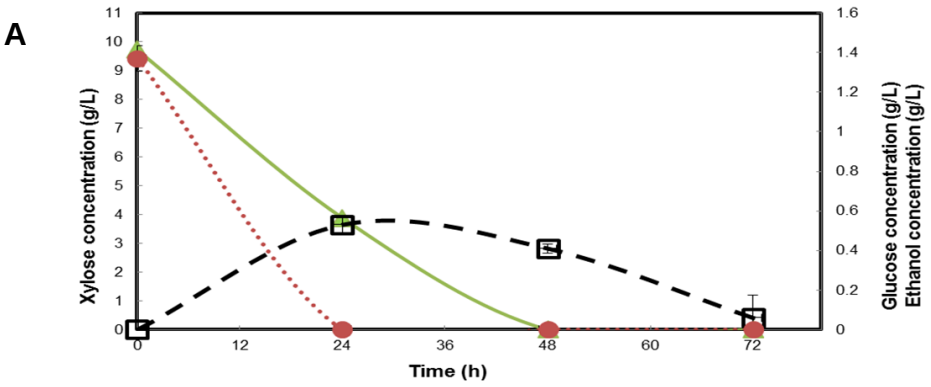


Fig. 2. Schematic summary of the electroporation-mediated genome shuffling between *K. marxianus* G2-16-1 and *P. stipitis* 10742^T

Under oxygen-limited conditions, *K. marxianus* G2-16-1 gave its maximum ethanol yield of 0.27 g/L from the xylose fermentation medium after 48 h (0.005 g ethanol/g xylose), whereas *P. stipitis* JCM 10742^T yielded an amount 7 times higher, at 1.9 g/L (0.38 g ethanol/g xylose). *K. marxianus* G2-16-1 fermented xylose to ethanol more effectively (by a factor of 10.1) under the aerobic condition than under the oxygen-limited condition, with a maximum ethanol yield of 2.73 g/L (0.05 g ethanol/g xylose). Likewise, *K. marxianus* S1.17 produced a higher (by a factor of 1.5) amount of ethanol from xylose under the aerobic condition (2.93 g/L at 40 °C for 48 h) than under the oxygen-limited condition. Prolonging the incubation time from 48 h to 72 h increased the ethanol production level by *K. marxianus* S1.17 to 3.29 g/L, but this decreased to 2.26 g/L after 84 h, while for *K. marxianus* G2-16-1, the ethanol yield decreased to 2.05 and 1.94 g/L after 72 and 84 h, respectively. Thus, the fermentation of the acid-treated leaf hydrolysate by *K. marxianus* S1.17 was performed under the aerobic condition for 72 h.

Ethanol Production from the Acid-treated Leaf Hydrolysate

The acid-treated leaf hydrolysate contained 1.38 g/L glucose (0.023 g/g) and 9.71 g/L xylose (0.17 g/g), which was in broad agreement with a previous report on the treatment of sugarcane leaves with 5.28% (v/v) HCl at 95 °C for 187 min, yielding 1.68 g/L glucose (0.036 g/g) and 8.92 g/L xylose (0.172 g/g) in the hydrolysate (Moodley and Gueguim Kana 2015). The hydrolysate was then supplemented with yeast extract and peptone, adjusted to pH 5.0, and separately fermented to ethanol under aerobic conditions by *K. marxianus* G2-16-1 and *K. marxianus* S1.17. The maximum ethanol produced by *K. marxianus* G2-16-1 was 0.53 g/L at 24 h, while for *K. marxianus* S1.17 it was 1.04 and 1.62 times higher, at 0.55 g/L after 24 h and at 0.86 g/L after 72 h (Fig. 3).



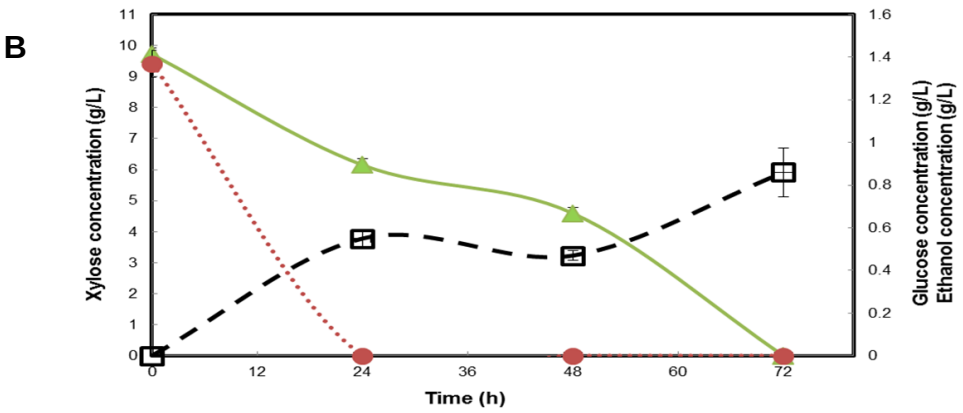
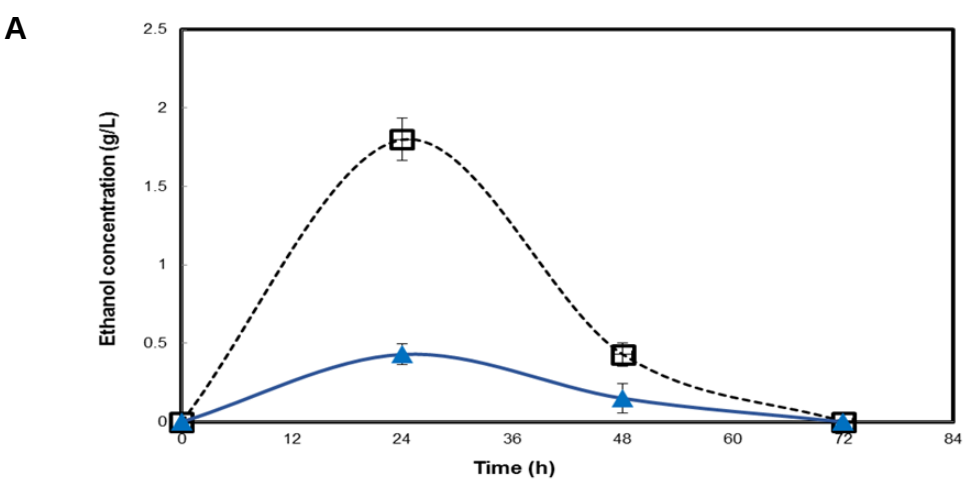


Fig. 3. Sugar utilization pattern and ethanol production level from acid-treated leaf hydrolysate by (A) *K. marxianus* G2-16-1 and (B) *K. marxianus* S1.17. Ethanol production (□), residual xylose (▲), and residual glucose (●). Data are shown as the mean ± 1SD and were derived from 3 independent repeated measurements.

Ethanol Production from Acid-treated Sugarcane Leaves

The hydrolysis of the acid-treated sugarcane leaves by various doses of Accellerase™ 1500 revealed that the minimal dose of Accellerase™ 1500 that released the highest amount of glucose (8.63 g/L) was 1,824 CMC units/g (data not shown).

The acid-treated sugarcane leaves, suspended in a citrate buffer and supplemented with yeast extract and peptone (pH 5.0), were fermented to ethanol using the SSF method under aerobic and oxygen-limited conditions at 40 °C by the addition of Accellerase™ 1500 (1,824 CMC units/g) with either *K. marxianus* G2-16-1 or *K. marxianus* S1.17 (final 10^8 cells/mL). Under aerobic conditions, *K. marxianus* S1.17 gave a maximum ethanol yield after 24 h of 1.8 g/L (0.0075 g/L/h), while *K. marxianus* G2-16-1 gave only 0.43 g/L (Fig. 4A). Under the oxygen-limited condition, *K. marxianus* S1.17 gave a maximum ethanol yield after 72 h of 4.73 g/L (0.08 g/g), which was 10% higher than that with *K. marxianus* G2-16-1 (Fig. 4B).



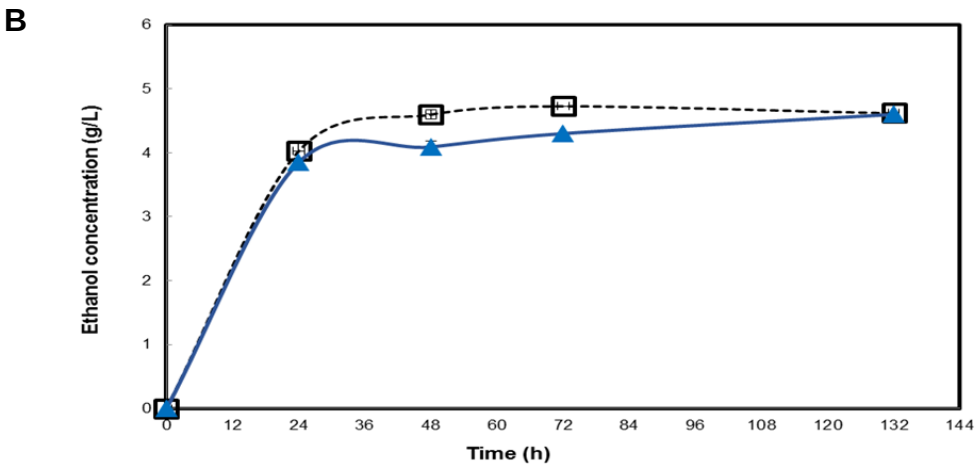


Fig. 4. Comparison of ethanol production from acid-treated sugarcane leaves by *K. marxianus* S1.17 (□) and *K. marxianus* G2-16-1 (▲) under an (A) aerobic or (B) oxygen-limited condition using the SSF process. Data are shown as the mean ± 1SD, derived from 3 independent repeats.

The results indicated that the capability of *K. marxianus* S1.17 to ferment xylose to ethanol was improved compared to the untransformed *K. marxianus* G2-16-1 (Figs. 3 and 4). The total ethanol produced by *K. marxianus* S1.17 was 5.59 g/L (0.095 g/g) from the acid-treated leaf hydrolysate (0.86 g/L) and from the acid-treated leaves using the SSF process (4.73 g/L). This was 23.5% higher than the amount of ethanol produced from the dilute sulfuric acid-treated sugarcane leaf slurry by *S. cerevisiae* using separate hydrolysis and fermentation (Jutakanoke *et al.* 2012). The low ethanol level obtained from fermentation of acid-treated leaf hydrolysate (pretreatment hydrolysate) and acid-treated sugarcane leaves under aerobic condition (Fig. 3 and Fig. 4A) might be a result of an ethanol vaporization. After 12 generations, glucose and xylose fermentation capabilities of the *K. marxianus* S1.17 were stable. Dawson and Boopathy (2007) reported an ethanol production yield of 0.01 g/g from sulfuric acid-treated sugarcane leaves by cellulose-fermenting *S. cerevisiae*. The maximum ethanol production level from sugarcane leaves treated with alkaline peroxide (121 °C, 15 min) and then co-hydrolysed by *Trichoderma reesei* cellulase (40 FPU/g) and *Aspergillus niger* ß-glucosidase (50 units/g) with fermentation by *S. cerevisiae* NRRL-Y-132 using the SSF process was 0.22 g/L after 72 h (ethanol productivity, 0.0031 g/L/h) (Hari Krishna *et al.* 1998). The replacement of the *S. cerevisiae* NRRL-Y-132 with the thermotolerant yeast, *Kluyveromyces fragilis* NCIM 3358, in the SSF process increased the ethanol yield to 0.28 g/L, giving an ethanol productivity of 0.0039 g/L/h (Hari Krishna *et al*. 2001).

CONCLUSIONS

1. Genome shuffling between *Kluyveromyces marxianus* G2-16-1, a cellobiase-producing yeast, and *Pichia stipitis* JCM 10742^T gave transformant, *K. marxianus* S1.17, which increased in xylose fermentation activity.
2. Ethanol tolerance could be used as criteria to screen for genome-shuffling mediated *K. marxianus* transformant which increased in xylose fermentation capability.

3. The total ethanol production from sugarcane leaves of *K. marxianus* S1.17 was 5.59 g/L (0.095 g ethanol/g sugar cane leaves).

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