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Characterization and ethanol fermentation of *Pichia* and *Torulaspora* strains

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ABSTRACT

Seventy-two yeasts were isolated from sugarcane juices and sugar process-sediments collected in Thailand by incubated at 30 and 40°C in oxygen-limited condition. Six and eleven isolated yeasts produced ethanol with 0.46 and 0.22 g/g initial glucose at 30 and 40°C, respectively. The highest ethanol production efficacy, 0.30 g/g glucose of maximum ethanol yield at 40°C was found in isolates G1-4(1) and G1-12(3). On the basis of their phenotypic characteristics including the D1/D2 region of the large-subunit ribosomal (LSU) of 26S rRNA gene sequence analysis, the isolates G1-4(1) and G1-12(3) were identified as *Pichia kudriavzvii* and *Torulaspora globosa*, respectively.

INTRODUCTION

Due to the world energy crisis and global warming problem, bio-ethanol which is renewable and green energy become more interesting. To make ethanol production cost competitive with gasoline, the use of lignocellulose which is abundant and low cost as raw material was extensive studied. Lignocellulose consists of 3 major components; cellulose, hemicellulose and lignin. Hydrolysis of the cellulose results in mainly glucose, a fermentable sugar. Because the cellulose component in ligno-cellulose is wrapped by hemicellulose and lignin. Therefore, ethanol production from lignocellulosic substrate is composed of three main steps; 1) Pretreatment: to unshield cellulose from hemicellulose and lignin, help cellulose to be more accessible to cellulase (Palonen *et al.*, 2004; Prasad *et al.*, 2007), convert crystalline cellulose to amorphous cellulose which is easier to be hydrolyzed by cellulose. 2) Saccharification (cellulose hydrolysis): to hydrolyze cellulose to glucose. Complete hydrolysis of cellulose is conducted by three types of enzymes (endoglucanase, exoglucanase and β-glucosidase) which work synergism (Turner *et al.*, 2007). The synergistic action is controlled by end product inhibition resulted in the decrease of final end product, glucose (Béguin, 1990). 3) Fermentation: resultant glucose from the saccharification step is fermented to ethanol by microorganism (Zheng *et al.*, 2009). Simultaneous saccharification and fermentation (SSF) is an ethanol fermentation process developed to solve the problem of end product inhibition of cellulose hydrolysis by immediately ferment glucose liberated from cellulose to ethanol. Advantage of the SSF process are reduction of fermentation time, contamination and production cost (Wingren *et al.*, 2003; Saha *et al.*, 2011). However, in the SSF process neither the cellulose hydrolysis nor ethanol fermentation is operated at its optimal condition due to difference in optimal temperature. Raising of optimal fermentation temperature closer to optimal temperature for cellulase activity (50°C), by using thermotolerant ethanol fermenting yeasts is a method to improve lignocellulosic ethanol yield produced by the SSF process.

This study deals with isolation and screening of high efficient ethanol fermenting yeasts. The yeasts selected were identified based on phenotypic characteristics, biochemical tests and including D1/D2 region of the large-subunit ribosomal of 26S rRNA gene sequence analysis.

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MATERIALS AND METHODS

Samples and yeast isolation

Twenty eight samples of sugarcane juice and sugar producing process-sediment were collected from sugar factory (Rajbury Sugar Co., Ltd.), Thailand. One g or 1 mL of the sample was inoculated into 5 mL of isolation medium (glucose 1%, yeast extract 0.3%, peptone 0.3%, chloramphenical 0.01% (w/v), and ethanol 3% (w/v), pH 5.0) (Laopaiboon *et al*., 2009) in 16 x 150 mm test tube and incubated at 30°C and 40°C, oxygen-limited condition for 7 days. Obtained cultures were purified by streak plate method using YPD agar medium (glucose 10%, yeast extract 0.3%, peptone 0.3% and agar 2% (w/v), pH 5.0) and incubated at 30°C and 40°C, oxygen-limited condition for 7 days. Oxygen-limited condition was performed by Candle jar method (Kumar, 2012). Resultant cultures were kept on YPD agar slant at 4°C.

Screening for ethanol fermenting yeast

Single colony of the isolated yeasts grown on YPD agar medium at 30°C or 40°C for 48 h was inoculated into 50 mL of fermentation medium (glucose 15%, yeast extract 0.6%, and peptone 0.9% (w/v), pH 5.0) in 250 mL Erlenmeyer flask and incubated at 30°C or 40°C, 200 rpm for 24 h. The culture transferred at 1% (v/v) to 50 mL of fresh fermentation medium in 250 mL Erlenmeyer flask and incubated at the same condition was used as inoculum.

The inoculum was inoculated at 10% (v/v) to fermentation medium (42.5 mL in 50 mL Erlenmeyer flask) and incubated at 30°C or 40°C, oxygen-limited condition for 48 h. Resultant culture were centrifuged at 4 °C, 12,000 rpm, for 5 min. Supernatants were analyzed for ethanol concentration by gas chromatography (Hewlett-Packard, HP 5890 Series, USA) with Porapak QS (Cabowax 20M) column (2m x 0.32m) at a temperature of 175 °C and a flame ionization detector (FID) at 150 °C. Helium, with a flow rate of 35 mL/min, was used as carrier gas (Jutakanoke *et al*., 2012).

Identification Methods

Phenotypic characterization

Vegetative cell, ascospore and colony morphologies of the isolated yeasts grown on YM agar and 5% malt extract agar were observed and compared with type strain as described in The Yeast: a taxonomic study, 4th ed (Kurtzman and Fell, 1998). For pseudohyphae detection, the Calmau plate culture method with corn meal agar was used (Kurtzman and Fell, 1998). Carbon assimilation test was performed by API kit ID 32 C (Biomerieux SA, France). Two days old cells suspended in ultra-pure water to final 2 McF (250 µl) were inoculated into C-medium, then transferred (135 µl) into well and incubated at 29±2 °C. Cell turbidity was monitored after 24, 48 and 72 h.

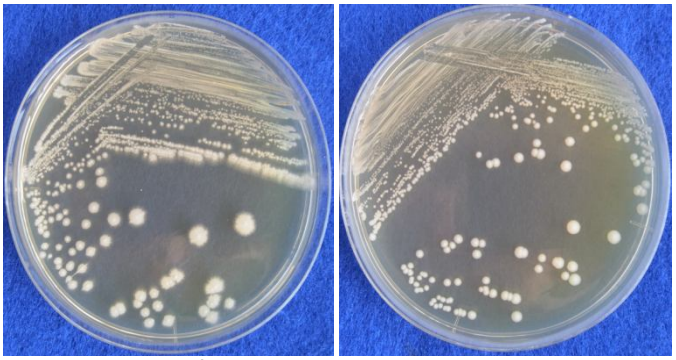
26S rDNA (D1/D2) sequencing and phylogenetic analysis

Yeast DNA was extracted by method of Manitis *et al*. (1982). D1/D2 domain of 26S rDNA was amplified by Polymerase Chain Reaction (PCR) and used F63 (5´-GCA TAT CAA TAA GCG GAG GAA AAG-3´) and LR3 (5´ GGT CCG TGT TTC AAG ACG-3´) as primers (Kurtzman and Robnett, 1998). Amplicon purified by Gel/PCR DNA Fragment Extraction Kit (Geneaid Biotech Ltd., Taipei, Taiwan) was directly sequenced by ABI PrismTM BigDyeTM Terminator Cycle sequence Ready Reaction Kit (Applied Biosystems, Stafford, USA) according to manufacturer's instruction. The resultant sequence was compared by BLASTn Homology Search (http://www.ncbi.nlm.nih.gov/blast). Generated sequence was aligned with 26S rDNA (D1/D2) sequence of related species using CLUSTAL X version 1.8 programs (Thompson *et al*., 1997). Phylogenetic tree was constructed from evolutionary distance data according to Kimura (1980) by the neighbor-joining method (Saitou and Nei, 1987). Bootstrap analysis (Felsenstien, 1985) was performed from 1,000 random re-samplings.

RESULTS AND DISCUSSION

Isolation and screening of ethanol fermenting yeast

A total of 72 yeasts, 35 strains were isolated at 30°C and 37 strains were isolated at 40°C. All isolates could ferment glucose to ethanol. Six yeasts isolated at 30 °C were found to produce ethanol at 30 °C higher than 0.46 g/g glucose whereas ten yeasts isolated at 40 °C could produce ethanol at 40°C higher than 0.22 g/g glucose (Figure 3). *Saccharomyces cerevisiae* TISTR 5596, a high ethanol producing control strain, produced ethanol 0.46 and 0.22 g/g glucose, at 30 °C and 40 °C, respectively. Isolates, G1-4(1) and G1-12(3), which produced the highest ethanol (0.30 g/g glucose) at 40°C were further identified.



A B

Fig. 1: Colonial appearance of isolates G1-4(1) (A) and G1-12(3) (B) on 5% malt extract agar after 3 days at 25°C.

Identification of isolates G1-4(1) and G1-12(3)

Morphological characteristics and biochemical tests of the 2 highest ethanol producing yeasts, G1-4(1) and G1-12(3) were examined. The isolate G1-4(1) formed butyrous and light-cream colored colonies (Figure 1) on 5% malt extract agar after 3 days at 25 °C. Cells are ovoid to elongate, and occur singly or in pairs. This strain showed similar assimilation results to the type strain of *Pichia kudriavzevii* except for glucosamine assimilation (Table 1). D1/D2 LSU sequence analysis showed 99% similarity to *Pichia kudriavzevii* Y-5396^T (Figure 2). Therefore, isolate G1-4(1) was identified as *Pichia kudriavzevii* (Kurtzman *et al*., 2011). The isolate G1-12(3) formed butyrous, dull to glistening, and tannish-

white colored colonies (Figure 1) on 5% malt extract agar after 3 days at 25 ^{o}C. Cells are spherical to ovoid, and occur singly or in pairs. It showed the same assimilation as the type strain of Torulaspora globosa (Kurtzman et al., 2011) (Table 1). The D1/D2 LSU sequence analysis showed 99% similarity with *Torulaspora globosa* Y-126^{T} (Figure 2). Pairwise comparison of 573 nucleotides with *T. globosa* Y-126^{T}, 1 nucleotide (nt) substitution was found. Therefore, isolate G1-12(3) was identified as *Torulaspora globosa* (Kurtzman *et al*., 2011). Recently, ethanol production from alkali-treated rice straw via simultaneous saccharification and fermentation of newly isolated thermotolerant *Pichia kudriavzevii* HOP-1 was reported. It produced maximum ethanol 0.42 g/g glucan or 0.24 g/g biomass at 40 ^{o}C after 24 h (Oberoi *et al.,* 2012). Yuangsaard *et al.* (2013) reported that newly isolated thermotolerant *Pichia kudriavzevii* could produce maximum ethanol (0.43 g/g glucose) from cassava starch hydrolysate at 40 ^{o}C within 24 h. The higher ethanol yield reported compared to this work might be a result of well controlled fermentation conditions, pH and temperature including agitation along the whole process, of Oberoi *et al.* (2012), and supplementation of nutrients $(NH_4)_2SO_4$ 0.05%, yeast extract 0.09%, KH_2PO_4 0.05%, $MgSO_4.7H_2O$ 0.05% (w/v)) in the ferment of Yuangsaard *et al.* (2013). Ethanol production by *Torulaspora globosa* at 40^{o}C was reported by Ngo Thi Phuong *et al*. (2010). Maximum ethanol produced was 0.26 g/g glucose which was lower than this study (0.30 g/g glucose). The result indicated *Torulaspora globosa*, G1-12(3), was a high efficient thermo-tolerant ethanol fermentating yeast.

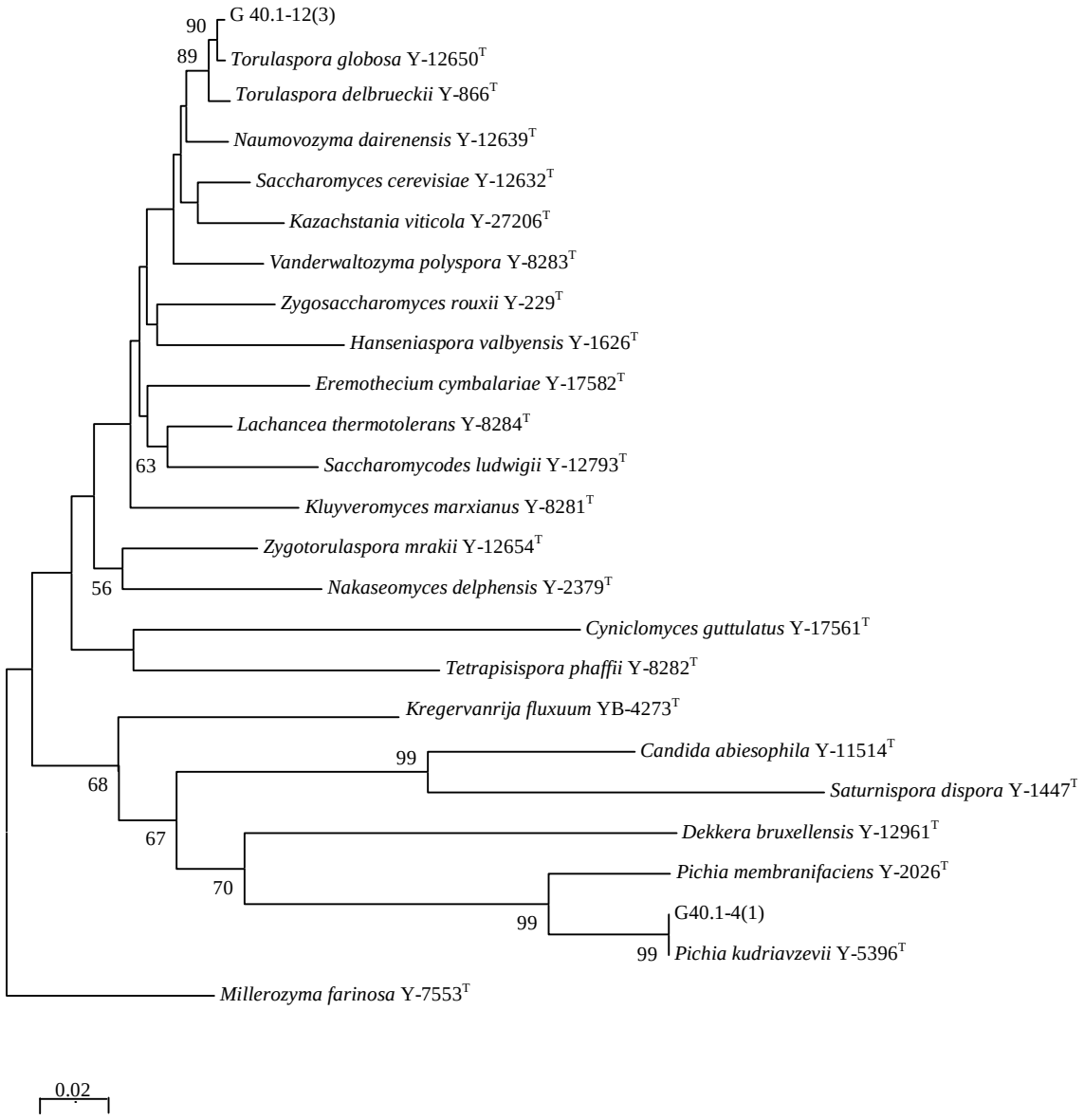


Fig. 2: Phylogenetic tree constructed by the neighbor-joining method based on D1/D2 domain of LSU rRNA gene sequences indicates position of isolates G1-4(1) and G1-12(3).

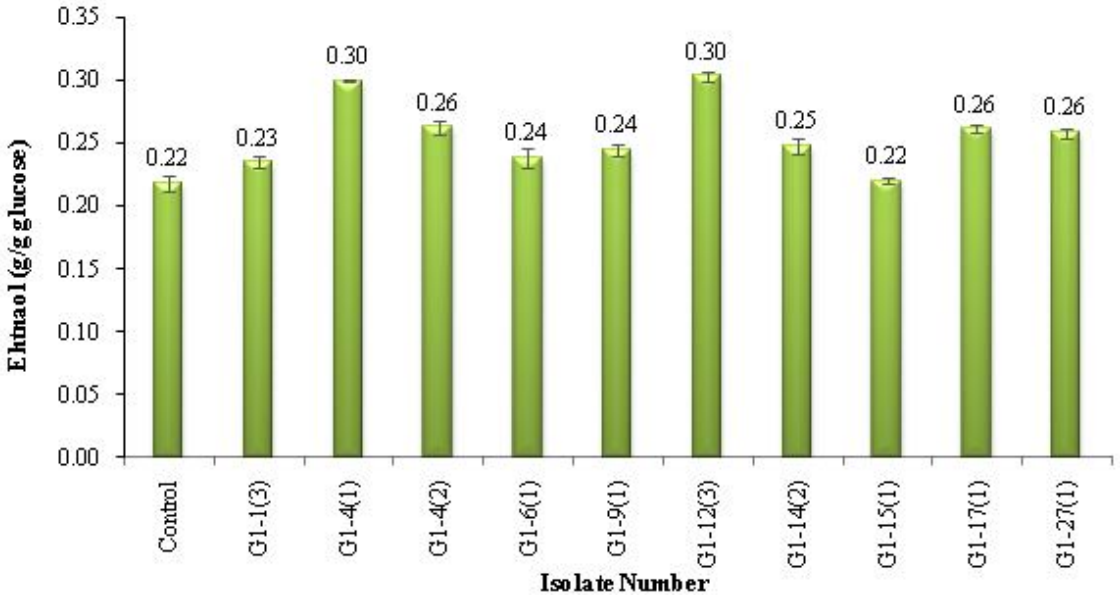


Fig. 3: Ethanol production of yeast isolates at 40°C.

Table. 1: Morphological and physiological characteristics of isolates G1-4(1) and G1-12(3).

Characteristics	G1-4(1)	PK[a]	G1-12(3)	TG[a]
Ascospore[b]	+[c]	+[c]	+[d]	+[d]
Pseudohyphae	+	+	+	+
Cycloheximide[e]	-	Nd	-	-
Esculin	+	Nd	+	Nd
D-Glucose	+	+	+	+
Glucosamine	-	+	-	-
Glycerol	+	+	+	v
N-acetyl- glucosamine	+	+	w	-
Lactic acid	+	+	+	+
Potassium gluconate	-	Nd	-	w/-
Potassium 2-keto gluconate	-	-	+	+
D-Mannitol	-	-	+	+
D-Raffinose	-	-	+	+
D-Sucrose	-	-	+	+
D-Trehalose	-	-	-	v

PK, *Pichia kudriavzevii*; TG, *Torulaspora globosa*; +, strong positive; d, delayed positive; w, weakly positive; v, variable; -, negative reaction; Nd, not determined. All are negative for assimilation of arabinose, D-cellobiose, erythritol, D-galactose, lactose, levulinic acid, D-maltose, D-melibiose, D-melezitose, methyl-α-D-glucoside inositol, palatinose, D-ribose, D-rhamnose, sodium glucuronate, D-sorbitol, L-sorbose and D-xylose, [a]Kurtzman *et al.* (2011). [b]Ascospore shape [c]Spherical and smooth ascospores.[d]Spherical fainty and roughened ascospores. [e]0.1 g/100 mL Cycloheximide for type strains.

CONCLUSIONS

Six and eleven high efficient ethanol fermenting yeasts were successfully isolated at 30°C and 40°C, respectively. Isolate G1-4(1) and G1-12(3) which produced the highest ethanol (0.30 g/g glucose) at 40 °C after 48 h were identified as *Pichia kudriavzvii* and *Torulaspora globosa*, respectively based on phenotypic characterization and D1/D2 region of large-subunit ribosomal (LSU) of 26S rRNA gene sequence analysis. The *Torulaspora globosa*, G1-12(3), showed higher ethanol producing efficiency than those of previously *Torulaspora globosa* reported.

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