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Diversity of Yeasts and Their Ethanol Production at high Temperature

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ABSTRACT

Thirty one yeasts isolated from sugarcane juice and process-sediments collected from different area of sugar factory were screened for ethanol production at 40°C. The isolates were belonged to 7 genera, namely *Pichia*, *Ogataea*, *Millerozyma*, *Meyerozyma*, *Candida, Kluyveromyces* and *Saccharomyces* based on phylogenetic analysis of D1/D2 region of the large subunit ribosomal (LSU) RNA gene. The majority of the isolates were identified as *Pichia kudriavzevii* (Group IB, 14 isolates), *Ogataea polymorpha* (Group II, 5 isolates), *Kluyveromyces maxianus* (Group VI, 5 isolates), *Pichia manshurica* (Group IA, 3 isolates), while the minor isolates were *Candida tropicalis* (Group V, 1 isolate), *Millerozyma farinosa* (Group III, 1 isolate), *Meyerozyma caribbica* (Group IV, 1 isolate) and *Sacharomyces cereviseae* (Group VII, 1 isolate). The isolates produced ethanol ranged from 2.97 to 57.10 g/L at 40°C. Isolates G3-9(1) and G3-3(1), identified as *K. marxianus* and *S. cerevisiae* showed high potential for ethanol production, 57.10 ± 0.23 and 49.42 ± 0.34 g/L, respectively.

INTRODUCTION

Yeasts are mostly unicellular organisms and can be classified either Ascomycetes or Basidiomycetes. They were distributed in varieties of habitat such as soils, plant surfaces and sugar rich samples including fruits, nectar and sap. Currently, there are approximately 1,500 recognized yeast species listed in the latest edition of The Yeasts (Kurtzman *et al*., 2011). Yeasts are huge potential in biotechnology for their enable to produce numerous valuable products such alcoholic beverages, food, feed and their applications in chemical, pharmaceutical industries and bioremediation (Satyanarayana and Kunze, 2009; Trama *et al*., 2014).

Increasing demand of petroleum consumption, depletion of energy reserves and concerning on environmental problems are promoting an effort to develop renewable and sustainability biofuel. Several attempts have been initiated to explore new alternatives energy friendly substances. Bioethanol received considerable attention as potentially renewable energy source. It was used to blend or substitute of gasoline due to its high octane number and low emission toxic substances. Bioethanol can produce through microbial fermentation process with high efficiently. At first, bioethanol production was developed using sugar or starchy based substrates such as molasses or cassava as a raw materials and *S. cerevisiae* has been common used as microbial agent for ethanol production at industrial level (Tesfaw *et al*., 2014). *S. cerevisiae* strains have several advantages such as high ethanol yield, high tolerance to ethanol and chemical inhibitors however they could produce high ethanol concentration at moderated temperature (25-35°C) (Aditiya *et al*., 2016).

Exploration, identification and characterization of other potential microorganisms that can produce ethanol at high temperature should be conducted to increase the industrial attractiveness for tropical countries and can apply to simultaneous saccharification process for reducing product inhibition and production costs (Abdel-Banat *et al*., 2010; Zhu *et al*., 2012). This study deals with the isolation and characterization of yeasts based on their phenotypic characteristics and phylogenetic

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analysis of D1/D2 region of the large subunit ribosomal RNA gene (D1/D2 LSU) including the ethanol production at higher temperature.

MATERIALS AND METHODS

Sources and isolation methods

Seventeen samples of sugarcane juice and process-sediments, collected from Thai Sugar industry Co., Ltd, in Kanchanaburi province, Thailand were used for yeast isolation. One gram or 1 mL of sample was enriched in 5 mL of GYPE medium (2% glucose, 0.3% yeast extract, 0.3% peptone, 0.1% chloramphenicol (w/v) and 3.0% ethanol (v/v), pH 5.6) in 16 × 150 mm test tubes and incubated at 40°C under oxygen limiting condition by wrapping cotton plug of the test tubes tightly with parafilm tape for 72 h. The isolates were re-streaked for purification on GYP agar plates containing 1% glucose, 0.3% yeast extract, 0.3% peptone, 2% agar and supplement with 0.1% chloramphenicol (w/v), pH 5.6 and incubated at 40°C, 48 h under oxygen limiting condition where oxygen were limited using candle jar. The yeast cultures were kept on yeast extract-peptone-dextrose (YPD) agar [1% yeast extract, 2% peptone, 2% glucose 2% agar (w/v), pH 5.6] at 4°C for further study.

Identification methods

Phenotypic characterization

Morphological and cultural characteristics of the isolates were investigated on YPD agar plate after incubated under aerobic conditions at 40°C for 48 h (Kurtzman *et al*., 2011). Carbon assimilation of the isolates was determined using cells grown on YPD agar at 30°C for 48 h. Cells of each isolates were suspended in 2 mL normal saline solution and adjusted the turbidity to obtained 2 McFarland units and 250 µL of the suspension was then transferred into 7 mL API C medium and mixed gently. The mixture of cell suspension and API C medium (135 µL) was inoculated into each cupule of ID32C kit (bioMérieux) and incubated at 30°C for 24-48 h. Turbidity of cell grown in ID 32C kit was determined as positive (+) and negative (−) compared to the control.

Genotypic characterization

The DNA extraction of cells grown on YPD agar at 30°C 48 h was carried out according to the procedure described by Lachance *et al*. (1999). PCR genomic DNA amplification was performed using primers pair NL1 (forward primer: 5'-GCATATCAA TAAGCG GAGGAAAAG-3') and NL4 (reverse: 5' GGT CCGTGT TTCAAGACG 3') as described by Kurtzman and Robnett (1998; 2003). Amplified PCR of LSU D1/D2 product was purified by ion Kit (Geneaid Biotech Ltd., Taipei, Taiwan). Agarose gel electrophoresis was performed to resolve the amplified using a standard molecular weight marker 100 bp DNA ladder (Fermentas, USA) as DNA marker. The gel was stained with ethidium bromide, visualized under UV light. The purified PCR products were sequenced using ABI Prism™ Big Dye™ Terminator Cycle sequence Ready Reaction Kit (Applied Biosystems, Stafford, USA) following the manufacturer's instructions. Sequencing was done with the same primers as used in the PCR reaction. The resultant LSU D1/D2 sequences were edited manually using BioEdit v7.2.5 (Hall, 1999). The LSU D1/D2 sequences were compared to those online data base available in National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast) by Blastn program and were aligned with muscle where gaps and missing data was deleted manually (Edgar, 2004). Neighbor-joining (NJ) phylogenetic tree was constructed using MEGA 7 (Kumar *et al*., 2016). The Kimura-2-parameter model was used to calculate the nucleotide-sequence divergence, and bootstrap values were obtained from 1000 replications (Kimura, 1980; Felsenstein, 1985).

Ethanol production

The isolated yeast grown on YPD at 40°C for 48 h was inoculated into 250 mL Erlenmeyer flask containing 50 mL of fermentation medium [15% glucose, 0.6% yeast extract and 0.9% polypeptone (w/v), pH 5.0] and incubated on rotary shaker (200 rpm) at 40°C for 24 h. One percent of each cultures was inoculated into 250 mL flask containing 50 mL of fresh fermentation medium, and incubated for 24 h under the above conditions then transferred 10% of the cultures into 42.5 mL of the fresh fermentation medium in 50 mL Erlenmeyer flask and incubated at 40°C for 48 h under oxygen-limiting condition. The cultures were then collected by centrifugation at 5000 rpm. The ethanol concentration of the above supernatants was determined by Gas Chromatography as described by Jutakanoke *et al*. (2014).

RESULTS AND DISCUSSION

Isolation and identification of isolates

Thirty one yeast strains were isolated from 17 sugar rich samples collected from different locations in sugar factory (Table 1). They were divided into four major groups and another three minor groups. All isolates were differentiated based on their morphological and cultural characteristics, carbon assimilation ability and D1/D2 region of the large subunit ribosomal (LSU) RNA gene analysis (Tables 1 and 2, Figure 1).

Group I consisted of 17 isolates, G3-1, G3-2 (2), G3-2 (3), G3-4 (1), G3-4 (2), G3-4 (4), G3-6 (11), G3-7, G3-10 (2), G3-12 (1), G3-13 (5), G3-14 (1), G3-14 (2), G3-16 (1), G3-16 (2), G3-17 (1) and G3-17 (3). Cells of isolates G3-1, G3-12 (1), G3-14 (1) were ovoid to cylindrical while colonies were tannish white, smooth, dull and butyrous.

They assimilated N-acetylglucosamine and glucosamine as sole carbon source and showed positive reaction with esculin. Based on 100% similarity of D1/D2 LSU sequences, isolates G3-1, G3-12(1), G3-14(1) were identified as *Pichia manshurica* (Group IA) (Table 1). Some *Pichia* strains were reported as non-*Saccharomyces* wine yeast such as *P. farinosa*, *P. kluyveri* and *P. terricola* (Jolly *et al*., 2006). Whereas *P. manshurica* was listed as the yeast that was causing of wine spoilage from their capable to produce volatile phenols (Saez *et al*., 2011). In addition, *Pichia* strains were the predominant species found in distilleries (Ubeda *et al*., 2014). The remained 14 isolates were identified as *P. kudriavzevii* (Group IB) (Table 1) based on 99-100% D1/D2 LSU sequence similarities. Their cells were ovoid to elongate while colonies were butyrous and light-cream colored. They assimilated N-acetylglucosamine,

lactic acid, glycerol and glucose. The thermotolerant yeast, *P. kudriavzevii* (synonymously known as *Issatchenkia orientalis*) was frequently isolated from foods and fruits. In addition, this species had a high potential to produce phytases, a useful enzymes in food processing and agriculture (Chan *et al*., 2012). Group II contained isolates G3-2(1), G3-4(3), G3-5(2), G3-10(3), G3-17(2) (Table 1).

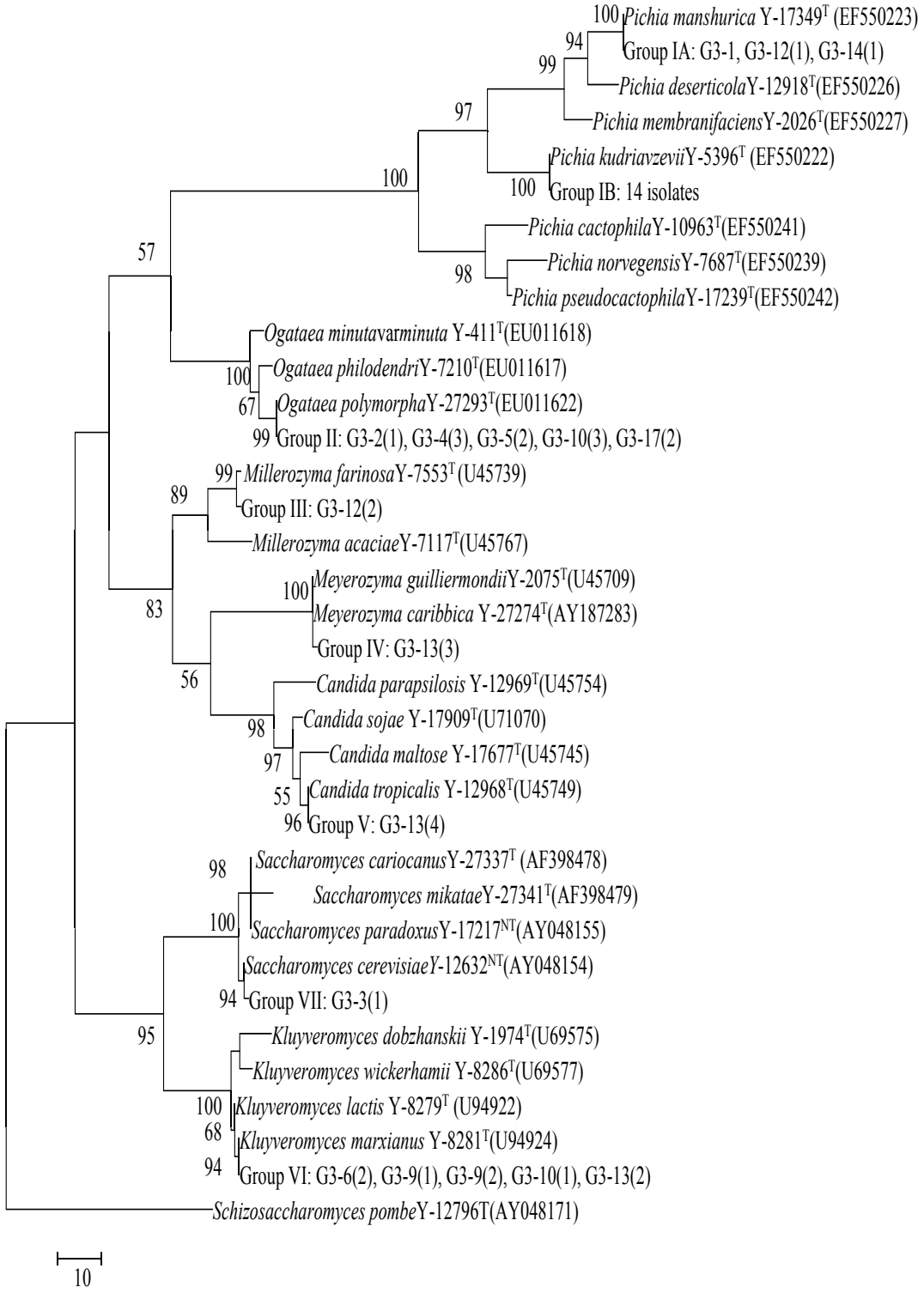

Fig. 1: Phylogenetic tree constructed using the neighbor-joining method showing the position of isolates and related species based on D1/D2 region of the large subunit ribosomal RNA gene sequences.

Table 1: Source, isolate number, Group, ethanol production, D1/D2 LSU ribosomal RNA gene sequence similarity (%) and identification.

Sources	Isolate no.	Group	Ethanol (g/L)	% Similarity	Identification
Waste sediment	G3-1	IA	2.97 ± 0.25	100	*P. manshurica*
Waste sediment	G3-12(1)	IA	3.00 ± 0.17	100	*P. manshurica*
Sugarcane juice (milling drum 1)	G3-14(1)	IA	3.37 ± 0.25	100	*P. manshurica*
Washing water (milling drum1)	G3-2(2)	IB	22.51 ± 0.26	100	*P. kudriavzevii*
Washing water (milling drum1)	G 3-2(3)	IB	29.72 ± 0.38	100	*P. kudriavzevii*
Sugarcane juice	G3-4(1)	IB	33.09 ± 0.25	100	*P. kudriavzevii*
Sugarcane juice	G3-4(2)	IB	25.88 ± 0.41	99	*P. kudriavzevii*
Sugarcane juice	G3-4(4)	IB	23.76 ± 0.33	99	*P. kudriavzevii*
Waste water	G3-6(1)	IB	31.55 ± 0.23	99	*P. kudriavzevii*
Sugarcane juice	G3-7	IB	28.80 ± 0.44	99	*P. kudriavzevii*
Filter cake	G3-10(2)	IB	22.42 ± 0.24	99	*P. kudriavzevii*
Waste water (clarifier tank)	G3-13(5)	IB	41.28 ± 0.33	99	*P. kudriavzevii*
Sugarcane juice (milling drum1)	G3-14(2)	IB	34.53 ± 0.15	100	*P. kudriavzevii*
Sediment, wet (boiler at 105°C)	G3-16(1)	IB	27.69 ± 0.32	100	*P. kudriavzevii*
Sediment, wet (boiler at 105°C)	G3-16(2)	IB	18.89 ± 0.25	100	*P. kudriavzevii*
Sediment, wet (boiler at 105°C)	G3-17(1)	IB	34.27 ± 0.25	99	*P. kudriavzevii*
Sediment, wet (boiler at 105°C)	G3-17(3)	IB	23.79 ± 0.27	100	*P. kudriavzevii*
Washing water (milling drum1)	G3-2(1)	II	16.16 ± 0.23	99	*O. polymorpha*
Sugarcane juice	G3-4(3)	II	12.42 ± 0.24	100	*O. polymorpha*
Waste water	G3-5(2)	II	12.74 ± 0.22	99	*O. polymorpha*
Filter cake	G3-10(3)	II	3.47 ± 0.15	100	*O. polymorpha*
Sediment, wet (boiler at 105°C)	G3-17(2)	II	7.36 ± 0.24	99	*O. polymorpha*
Waste sediment	G3-12(2)	III	24.99 ± 0.32	99	*M. farinosa*
Waste water (clarifier tank)	G3-13(3)	IV	19.87 ± 0.30	99	*M. caribbica*
Waste water (clarifier tank)	G3-13(4)	V	21.95 ± 0.11	100	*C. tropicalis*
Sediment, dry (boiler at 105°C)	G3-9(1)	VI	57.10 ± 0.23	100	*K. marxianus*
Sediment, dry (boiler at 105°C)	G3-9(2)	VI	32.01 ± 0.22	100	*K. marxianus*
Filter cake	G3-10(1)	VI	37.05 ± 0.23	100	*K. marxianus*
Waste water (clarifier tank)	G3-13(2)	VI	46.34 ± 0.24	100	*K. marxianus*
Waste water	G3-6(2)	VI	28.79 ± 0.5	100	*K. marxianus*
Sugarcane syrup	G3-3(1)	VII	49.42 ± 0.34	100	*S. cerevisiae*

Cells were spherical to elongate. Colonies were butyrous and white to cream colonies. The isolates assimilated D-trehalose, D-mannitol, D-xylose, D-ribose, glycerol, palatinose, erythritol, D-melezitose and D-glucose. They showed 99-100% similarity of D1/D2 LSU sequences to *Ogataea polymorpha* NRRL Y-5445^{T}. Therefore, they were identified as *O. polymorpha*.

This species formerly classified in the genus *Hansenula* and was thermotolerant microorganism that was able to ferment xylose, which presented in lignocellulosic raw materials. Therefore, they could be applied for biomass based bioethanol production at high temperature (Dmytruk *et al*., 2008).

Group III contained isolate G3-13(3) (Table 1). Cells were ovoid to elongate. Colonies were white to yellowish white in colored. The isolate assimilated N-acetylglucosamine, D-treharose, D-mannitol, D-ribose, D-glycerol, D-glucose and erythritol (Table 2). Based on 99% D1/D2 LSU sequence, it was identified as *Millerozyma farinosa* (syn. *Pichia* farinosa). *Millerozyma farinosa* has been reported as polyol protease enzyme and salt-mediated killer toxin producing strain (Suzuki *et al*., 2001). Group IV, contained isolate G3-12(2) (Table 1). Cells were ovoid to elongate. Colonies were smooth, circular, convex, white to ivory-white in colored, after grew on YPD agar at 40°C for 48 h. The isolate assimilated D-galactose, cyclohexamide, sucrose, N-acetylglucosamine, D-sorbitol, D-xylose and glycerol while D-raffinose, D-trehalose, Potassium-2-ketogluconate, D-glucose and mannitol were assimilated weakly. This stain was identified as *Meyerozyma caribbica* based on 99% D1/D2 LSU sequence similarity. *M. caribbica* was frequently isolated from fermented beverage and could ultilized xylose in hemicellulose hydrolysate with high efficiency (Weinhandl *et al*., 2014; Cassa-Barbosa *et al*., 2015; Papalexandratou and De Vuyst, 2011). Group V contained isolate G3-13(4) (Table 1). Cells were subglobose to ovoid while colonies were dull, smooth surface, soft and creamy, cream-colored or off white to grey. This strain assimilated

D-galactose, cyclohexamide, sucrose, N-acetylglucosamine, D-maltose, D-treharose, potassium-2-ketogluconate, methyl-α-D-glucopyranoside, D-mannitol, D-sorbitol, D-xylose, glycerol, palatinose, D-melezitose, D-glucose and glucosamine. Based on the analysis of D1/D2 LSU sequence similarity (100%), this strain was identified as *Candida tropicalis*. This species was thermotolerant yeast that could be growth in various kinds of substrates. It was reported as a useful microorganism for ethanol production from starch and lignocellulosic biomass (Oberoi *et al*., 2010; Jamai *et al*., 2007).

Table 2: Phenotypic characteristics of yeast isolates.

Characteristics	Group I		Group II	Group III	Group IV	Group V	Group VI	Group VII
	A	B						
Cell form	**OC**	**OE**	**SE**	**OE**	**OE**	**SO**	**GEC**	**GOE**
Colony color	**TW**	**LC**	**WC**	**WY**	**WI**	**C**	**LC**	**CB**
Carbon assimilation								
N-acetylglucosamine	+	+	−	+	+	+	−	−
D-Cellobiose	−	−	−(+1)	−	−	−	−	−
Cyclohexamide	−	−	+ (−1)	−	+	+	+	−
Erythritol	−	−	+	+	−	−	−	−
Esculin	+	w (+4)	+	+	+	w	+	w
D-Galactose	−	−	−	−	+	+	+	+
Glucosamine	+	−	−	−	−	+	−	−
Glycerol	−	+	+	+	+	+	+	w
methyl-α-D-Glucopyranoside	−	−	−	−	−	+	−	+
Lactic acid	−	+	−	−	−	−	+	w
Levulinic acid	−	+ (−7)	−	−	−	−	−	−
Palatinose	−	−	+	−	−	+	−	−
Potassium gluconate	−	−	−	w	−	−	−	−
Potassium 2-ketogluconate	−	−	−	−	w	+	−	−
D-Raffinose	−	−	−	−	w	−	+	+
D-Ribose	−	−	+	+	−	−	−	−
D-Maltose	−	−	+ (−1)	−	−	+	−	+
D-Mannitol	−	−	+	+	w	+	−	−
D-Melezitose	−	−	+	−	−	+	−	−
D-Sorbitol	−	−	+ (−2)	−	+	+	+	−
L-Sorbose	−	−(+3)	−	−	−	−	−	−
Sucrose	−	−	+ (−1)	−	+	+	+	
D-Trehalose	−	−	+	+	w	+	−	+
D-Xylose	−	+	−	−	+	+	+ (w1)	−

+, positive reaction; −, negative reaction; w, weakly positive. All strains assimilated glucose but did not assimilate L-arabinose, lactose, inositol, l-rhamnose, D-melibiose and sodium gluconate. Numbers in parentheses indicate the number of isolates showing the reaction. GEC, globose, ellipsoidal to cylindrical; GOE, globose, ovoid or elongate; OC, ovoid to cylindrical; OE, ovoid to elongate; SO, cells are subglobose to ovoid; SE, spherical to elongate. TW, Tannish white; LC, light-cream; WC, white to cream; WY, white to yellowish; WI, white to ivory; C, cream; LC, light cream; CB, cream colored to brown.

Group VI consisted of isolates G3-9(1), G3-9(2), G3-10(1), G3-13(2) and G3-6(2) (Table 1). Cells were globose, ellipsoidal to cylindrical while colonies were dull, flat and round, butyrous texture, cream colored to brown or rarely pink. These strains were identified as *Kluyveromyces marxianus* based on D1/D2 LSU sequence similarity (100%). *K. marxianus* was known as high biotechnological potential yeast species due to its ability to use a variety of substrates and high growth rate under aerobic conditions. *K. marxianus* was a generally recognized as safe (GRAS) yeast and could ferment ethanol with high efficiently at temperatures of 38-45°C. So this strain could be used with high potential to produce ethanol commercially by simultaneous saccharification and fermentation (SSF) to reduce production costs (Fonseca *et al*., 2008; Akaracharanya *et al*., 2016).

Group VII contained isolate G3-3(1). Cells were globose, ovoid or elongate while colonies were butyrous, flat and light cream colored. The isolate assimilated D-glucose, D-galactose, sucrose, D-raffinose, D-maltose, D-trehalose, methyl-α-D-glucopyranoside and eaculin (weakly) but did not assimilate glycerol and lactic acid. Based on D1/D2 LSU sequence similarity (99%), this strain was identified as *Saccharomyces* cerevisiae. *S. cerevisiae* was known for its preference towards glucose. It

quickly utilized glucose and used other preferred carbon sources after glucose depletion. *S. cerevisiae* lacks the ability to ferment pentose sugars so it could not use for bioethanol production from lignocellulosic biomass (Tesfaw *et al*., 2014; Weinhandl *et al*., 2014).

Ethanol production

All isolates were determined for ethanol production capacity at 40°C as shown in Tables 2. Ethanol production efficiency of Group IA, *P. kudriavzevii* was varied from 18.89 to 41.28 (g/l). *P. kudriavzevii* could produce more ethanol than the conventional *S. cerevisiae* at 40 and 45°C about 35 and 20%, respectively and cloud fermented ethanol in wide pH rages when compared with *S. cerevisiae* (Oberoi *et al*., 2012). However, group IB, *Pichia manshurica* showed very low ethanol production at 2.97-3.37 g/L. Ethanol production efficiency of *Ogataea polymorpha* (group II) were varied from 3.47 to 16.61 g/L. It was reported that *Ogataea polymorpha* (syn. *Hansenula polymorpha*) capable of fermenting xylose, cellobiose and glucose to ethanol at high temperatures (45°-50°C) (Ryabova *et al*., 2003). So it was counted as one of important yeast that could develop to produce ethanol from lignocellulosic biomass, despite the fact that ethanol yield of this strain still rather low. *Millerozyma farinosa* G3-13(3) (Group III) produced ethanol at 24.99 g/L whereas *Meyerozyma caribbica* G3-12(2) (group IV) produced ethanol at 19.87 g/L. Group V, *Candida tropicalis* G3-13(4) produced 21.95 g/L of ethanol from glucose. Group VI, *K. marxianus* could produce ethanol from glucose substrate range from 28.79-57.10 g/L at 40°C. In this study *K. marxianus* G3-9(1) showed the highest ethanol production (57.10 g/L). Group VII, *S. cerevisae* G3-3(1) produced 49.42 g/L of ethanol that was higher than TISTR 5597 (34.05 ± 0.18 g/L). *S. cerevisae* was one of important commercial yeast which generally used in beverage, ethanol production and bread making. This yeast species has several advantages on industrial ethanol production due to able to tolerate high ethanol concentration and produce hight ethanol concentration at moderated temperature (37°C or less) but it lacked an ability to assimilate xylose that could not be applied for lignocellulosic ethanol fermentation (Tesfaw *et al*., 2014).

CONCLUSION

In this present study, *Pichia kudriavzevi* was the predominant species in the sugar rich area followed by *Kluyveromyces maxianus*, *Pichia manshurica*, *Ogataea polymorpha*, *Candida troicalis*, *Millerozyma farinose*, *Meyerozyma caribbica* and *Sacharomyces cereviseae*. All isolates were valuated their ethanol fermentation ability and *K. marxianus* G3-9(1) and G3-3(1) and *S. cerevisiae* G3-3(1) showed high potential for ethanol production at high temperature (40°C).

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CONFLICT OF INTERESTS

There are no conflicts of interest.

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