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Isolation and characterization of thermotolerant ethanol-fermenting yeasts from Laos and application of whole-cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) analysis for their quick identification

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Thermotolerant yeasts, which are expected to be applicable for high-temperature fermentation as an economical process, were isolated from four provinces in Laos. Of these yeasts, five isolates exhibited stronger fermentation abilities in a 16% sugars-containing medium of glucose, sucrose, sugarcane or molasses at 40°C than that of *Kluyveromyces marxianus* DMKU 3-1042, one of the most thermotolerant and efficient yeasts isolated previously in Thailand. One of the five strains, BUNL-17, exhibited the highest ethanol fermentation performance at 45°C. Yeast identification was achieved by whole-cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) analysis as well as by nucleotide sequencing of the D1/D2 domain of the large subunit rRNA gene, revealing that the isolated strains can be categorized into *Pichia kudriavzevii*, *Cyberlindnera rhodanensis* and *K. marxianus* and that all of the five strains are *K. marxianus*. The results of this study showed that the former analysis is much faster than the latter and reliable and equivalent to the latter.

Key words: Ethanol fermentation, thermotolerant yeast, *Kluyveromyces marxianus*.

INTRODUCTION

Global energy demand has been continuously increasing due to an increase in the worldwide human population and economic growth. The demand is mostly supplied from traditional fossil fuels, causing a critical elevation of the greenhouse gas level in the Earth's atmosphere (Talebnia et al., 2010; Ballesteros et al., 2006). Bioethanol is one alternative for fossil fuels and has been widely utilized as a dominant biofuel. Worldwide production of

bioethanol has been increasing over the past 35 years, and the gross output of biofuels including ethanol in 2022 is forecasted to be more than 126 billion liters (Rees, 2014).

Considering the convention on biological diversity that includes access to genetic resources and benefit-sharing, useful microbes isolated from own country are beneficial in usability and industrial applicability for each country. Thermotolerant microbes are expected to be crucial for fermentation industries in tropical countries and even in non-tropical countries in summer, because they can be used for high-temperature fermentation, being stably achieved at temperatures around 40°C, which has several advantages including reduction of cooling cost, prevention of contamination and enhancement in enzyme reaction of hydrolysis (Murata et al., 2015). In such fermentation for ethanol production, it is necessary to acquire an efficient yeast strain with a strong tolerance to high temperatures. Mesophilic strains of *Saccharomyces cerevisiae*, however, have been used for a long time in industrial ethanol production. Although there are numerous reports on the potentials of thermotolerant yeast strains, there has been almost no application of them for industrial ethanol production (Kida et al., 1992; Morimura et al., 1997; Sree et al., 2000). The reason(s) is not clear, but it appears that there is still no suitable yeast available.

Many applications have been attempted to isolate thermotolerant yeasts that are capable of growing and fermenting ethanol at high temperatures. Banat et al. (1992) used an enrichment technique for obtaining thermotolerant, fermentative yeasts at 50°C in a medium containing 0.3% malt extract, 0.3% yeast extract, 0.5% peptone and 1% glucose. Limtong et al. (2007) enriched thermotolerant yeasts at 35°C in a medium containing 5 or 8% sugarcane juice, 0.05% $(NH_4)_2SO_4$ and 4% ethanol (pH 4.5), Yuangsaard et al. (2013) used a yeast extract dextrose (YPD) medium supplemented with 4% ethanol at 40°C and Ueno et al. (2001) isolated thermotolerant yeasts from hot spring drainage, Brooks (2008) isolated thermotolerant *S. cerevisiae* from ripe banana peels in Nigeria and Saini et al. (2015) isolated thermotolerant *Kluyveromyces marxianus* strain from local dairies in India. For obtaining thermotolerant yeasts that are applicable for industrial fermentation, the development of a simple and quick screening procedure including species identification is needed.

In this study, an enrichment procedure was applied at a relatively high temperature for screening of thermotolerant and ethanol-fermenting yeasts from samples collected in Laos. Attempt was also made to apply whole-cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) analysis as a very fast procedure for species identification compared to nucleotide sequencing of rRNA genes. The thermotolerance and ethanol fermentation ability of the isolated strains were compared with those of the *K. marxianus* strain DMKU 3-1042 as a control, which is one of the most thermotolerant and efficient strains isolated in Thailand (Limtong et al., 2007).

MATERIALS AND METHODS

Isolation of thermotolerant yeast strains

Yeasts were isolated from samples of fruits, vegetables, leaves, and soils in four provinces: Luang Phrabang, Xayabury, Xiengkhuang, and Vientiane of Lao People's Democratic Republic (PDR). Isolation was carried out at 37°C by an enrichment culture. Samples (5 to 10 g) of fruits pressed in small pieces, leaves cut in small portions and mashed soil were transferred into 100-ml Erlenmeyer flasks containing 10 ml of YPD medium containing 1% yeast extract (Difco), 2% peptone (Difco) and 2% glucose (Sigma-Aldrich), and incubated at 37°C for 3 days with occasional shaking. The cultures were then streaked on YPD agar plates and incubated at 37°C for 24 to 48 h. The first examination was carried out to test thermotolerance on agar plates of YPD and yeast extract peptone xylose (YPX), which contained 2% xylose (Wako Chemicals) instead of glucose, at different temperatures for 48 h.

Screening of ethanol-producing thermotolerant yeasts

Screening for the ability of ethanol fermentation at high temperatures was conducted at 40 and 45°C in 250-ml Erlenmeyer flasks containing 100 ml of YP medium with 16% glucose (YP + 16% D medium) or sucrose (Sigma-Aldrich) (YP + 16% S medium), 16% sugarcane juice supplemented with 0.05% $(NH_4)_2SO_4$, 0.5% KH_2PO_4 and 0.15% $MgSO_4 \cdot 7H_2O$ (pH 4.5) (16% sugarcane medium) and 16% molasses supplemented with 0.05% $(NH_4)_2SO_4$ (pH 4.5) (16% molasses medium). The pre-culture was prepared in YPD medium at 25°C for 18 to 24 h under a rotationally shaking condition at 160 rpm. The pre-culture was transferred at the rate of 5% to each culture medium, followed by incubation at an appropriate temperature under a rotationally shaking condition at 160 rpm.

Analysis of fermentation parameters

Cell growth was determined by measuring optical density at 660 nm on a spectrophotometer (Spectrophotometer 258, Corning, New York, USA) after washing twice with distilled water. Ethanol concentration was analyzed by a gas chromatography (Shimadzu GC-9A, Shimadzu, Kyoto, Japan) using polyethylene glycol (PEG-20 M) packed column (length 2.1 m, OD 5 mm, ID 3.2 mm), nitrogen as a carrier gas (35 ml/min), and a flame ionization detector (injection temperature at 200°C, oven temperature at 180°C, detector temperature at 200°C). Sugars as carbon source in media were analyzed by high-performance liquid chromatography apparatus (Hitachi, Japan) with a GLC610-S Gel pack column (Hitachi) connected to a refractive index detector Model L-2490 (Hitachi) in the mode of 0.5 ml/min eluent of deionized water at 60°C (Rodrussamee et al., 2011).

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Nucleotide sequencing of D1/D2 domain

Identification of isolated strains was carried out by determination of the nucleotide sequence of the D1/D2 domain in the large-subunit rDNA. The genomic DNA was extracted from yeast cells by the slightly modified method of Green and Sambrook (2012). Cells were grown in 3 ml of YPD medium at 30°C under a moderate agitation condition overnight, harvested by centrifugation (14,000 rpm) for 5 min, and resolved in 0.5 ml of sorbitol buffer (1 M sorbitol and 0.1 mM EDTA at pH 7.5). To the cell suspension was added 1 µl of zymolyase solution (5 U/µl, Zymo Research) and the suspension was incubated at 37°C for 30 min. Cells were then collected by centrifugation (14,000 rpm) for 1 min and resolved in 0.5 ml of yeast resuspension buffer (50 mM Tris-Cl and 20 mM EDTA at pH 7.5). To the resolved sample was added 50 µl of 10% SDS and the sample was incubated at 65°C for 30 min. To the incubated sample was added 0.2 ml of 5 M potassium acetate and the sample was kept on ice for 1 h. Cell debris was then removed by centrifugation (14,000 rpm) at 4°C for 5 min. From the supernatant, nucleic acids were recovered as a pellet by centrifugation (14,000 rpm) at 4°C for 5 min after the addition of an equal volume of isopropanol and storage at room temperature for 5 min. The pellet was suspended in 0.15 ml of TE buffer (pH 8.0) containing 30 µg/ml RNase A and incubated at 37°C for 30 min. DNA was then recovered as a pellet by centrifugation (14,000 rpm) for 5 min after addition of 30 µl of 3 M sodium acetate (pH 7.0) and 0.2 ml of isopropanol and storage at room temperature for 5 min. The pellet was dried and suspended in 30 µl of TE buffer, which was used for polymerase chain reaction (PCR) as genomic DNA. PCR was done with a set of forward primer NL-1 and reverse primer NL-4 (O'Donnell, 1993). The PCR products were subjected to agarose gel electrophoresis, purification with a QIA Quick Purification Kit (Qiagen, Ontario, USA) and cycle-sequencing with an ABI BigDye Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems, California, USA) with the primers NL-1 and NL-4. The nucleotide sequences of samples were then determined on an ABI PRISM 3100 automated DNA sequencer (Applied Biosystems, California, USA) and compared with those of type strains in databases using the BLAST homology search (Altschul et al., 1990). The nucleotide sequences were aligned using the multiple alignment program CLUSTAL_X version 2.1 (Thompson et al., 1997), and a phylogenetic tree was constructed from the evolutionary distance data with Kimura's two parameter correction (Kimura, 1980), using the neighbor joining method (Saitou and Nei, 1987) and the MEGA software version 6.0 (Tamura et al., 2013). Confidence levels of the clades were estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985).

Whole-cell MALDI-TOF/MS analysis

Whole-cell MALDI-TOF/MS analysis was performed as described previously (Tani et al., 2015). In brief, a loopful of well-grown yeast (2 to 3 days old, usually 5 to 10 mg in wet weight) on YPD agar plates was suspended in 300 µl of 75% ethanol. The suspension was centrifuged at 15,000 rpm for 2 min. The supernatant was discarded and 50 µl of 70% formic acid was added and mixed. Next, 50 µl of acetonitrile was added and mixed well again. One microliter of the sample was placed onto a spot of a MALDI steel target plate and dried in air. Then 2 µl of matrix solution (saturated solution of sinapinic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) was overlaid onto the sample, and the samples were dried in air.

The samples were analyzed with MALDI-TOF/MS (Ultraflex, Bruker Daltonics). Mass spectra were obtained using a positive linear mode in the range of mass-to-charge ratios (m/z) of 2,000 to 20,000. Protein standard comprised insulin ([M+H] = 5734.56), ubiquitin-I ([M+H]+ = 8565.89), cytochrome *c* ([M+H]+ = 12361.09 and [M+2H]2+ = 6181.05), and myoglobin ([M+H]+ = 16952.55 and [M+2H]2+ = 8476.77) (Bruker Daltonics).

The obtained spectrum data were further analyzed to create a dendrogram based on spectra similarity using BioTyper software (Bruker Daltonics) under the standard setting (Tani et al., 2015).

RESULTS

Isolation and selection of thermotolerant yeast strains

In total, 31 thermotolerant yeast strains that could grow on YPD plates at 40°C were isolated by enrichment culture at 37°C with 97 samples, which were collected in four provinces of Laos. Of these strains, 24, four, two strains and one strain were derived from fruits, vegetables, flowers, and soil, respectively. They were further subjected to growth capability tests on YPD and YPX plates at different temperatures (Table 1). DMKU 3-1042, which is one of most thermotolerant *K. marxianus* strains isolated in Thailand (Limtong et al., 2007), was used as a control. DMKU 3-1042 has been extensively analyzed (Rodrussamee et al., 2011; Lertwattanasakul et al., 2011) and its complete genome has been determined (Lertwattanasakul et al., 2015). As a result, nine strains grew well on YPD plates both at 45 and 48°C. On YPX plates, all strains could grow at 40°C, but only five strains grew well at 48°C.

All of the isolates were then used in the experiments to compare ethanol fermentation abilities. They were cultivated in YP + 16% D medium at 40°C under a rotationally shaking condition at 160 rpm (Table 2). BUNL-14, 15, 16, 17, 21, and 23 strains exhibited high levels of ethanol production both at 12 h (4.76 to 6.56% (w/v)) and 24 h (5.39 to 7.24% (w/v)), nearly equivalent to or more than that of DMKU 3-1042. Of these strains, BUNL-14 and 17 produced the highest levels of ethanol at 12 and 24 h, respectively. All of these strains except for BUNL-16 were strongly thermotolerant (Table 1). Therefore, five strains, BUNL- 14, 15, 17, 21, and 23, as relatively thermotolerant and highly efficient strains were further used in experiments on ethanol fermentation with various sugars.

Screening of thermotolerant yeasts for ethanol production at high temperatures

The five strains were cultivated in three media, YP + 16% S medium (Table 3), 16% sugarcane (Table 4) and 16% molasses (Table 5), at 40°C under a shaking condition. In the YP + 16% S medium, all of the strains except for BUNL-14 showed ethanol production higher than or equivalent to that of DMKU 3-1042 at 12 h, and BUNL-17 showed higher level of ethanol production than those of DMKU 3-1042 at 24 h. In the 16% sugarcane medium, all of the strains produced higher levels of ethanol than those of DMKU 3-1042 at 12 h, and BUNL-14, 15, 17,

Table 1. Growth of the isolated strains on YPD and YPX plates at different temperatures and their identification.

Strains	YPD[a] Temperature (°C)				YPX[a] Temperature (°C)				Identification	
	37	40	45	48	37	40	45	48	D1/D2[b]	TOF/MS[c]
BUNL-1	+++	+++	-	-	+	+	-	-	*P. kudriavzevii* (LC093941)[d]	*P. kudriavzevii*
BUNL-2	+++	+++	-	-	+	+	-	-	*P. kudriavzevii* (LC093942)	*P. kudriavzevii*
BUNL-3	+++	+++	-	-	+	+	-	-	NI	*P. kudriavzevii*
BUNL-4	+++	+++	-	-	+	+	-	-	*P. kudriavzevii* (LC093943)	*P. kudriavzevii*
BUNL-5	+++	+++	-	-	+	+	-	-	NI	*P. kudriavzevii*
BUNL-6	+++	+++	-	-	+	+	-	-	*P. kudriavzevii* (LC093944)	*P. kudriavzevii*
BUNL-7	+++	+++	-	-	+	+	-	-	NI	*P. kudriavzevii*
BUNL-8	+++	+++	-	-	+	+	-	-	*P. kudriavzevii* (LC093945)	*P. kudriavzevii*
BUNL-9	+++	+++	-	-	+	+	-	-	NI	*P. kudriavzevii*
BUNL-10	+++	+++	-	-	+	+	-	-	*P. kudriavzevii* (LC093946)	*P. kudriavzevii*
BUNL-11	+++	+++	-	-	+	+	-	-	*P. kudriavzevii* (LC093947)	*P. kudriavzevii*
BUNL-12	+++	+++	-	-	++	++	-	-	*C. rhodanensis* (LC093948)	*C. rhodanensis*
BUNL-13	+++	+++	+++	+++	+++	+++	+++	+++	*K. marxianus* (LC093949)	*K. marxianus*
BUNL-14	+++	+++	+++	+++	+++	+++	++	++	*K. marxianus* (LC093950)	*K. marxianus*
BUNL-15	+++	+++	+++	+++	+++	+++	++	++	*K. marxianus* (LC093951)	*K. marxianus*
BUNL-16	+++	+++	-	-	+	+	-	-	NI	*P. kudriavzevii*
BUNL-17	+++	+++	+++	+++	+++	+++	+++	++	*K. marxianus* (LC093952)	*K. marxianus*
BUNL-18	+++	+++	-	-	+	+	-	-	NI	*P. kudriavzevii*
BUNL-19	+++	+++	-	-	+	+	-	-	NI	*P. kudriavzevii*
BUNL-20	+++	+++	-	-	+	+	-	-	NI	*P. kudriavzevii*
BUNL-21	+++	+++	+++	+++	+++	+++	+++	+++	*K. marianus* (LC093953)	*K. marxianus*
BUNL-22	+++	+++	-	-	+	+	-	-	NI	*P. kudriavzevii*
BUNL-23	+++	+++	+++	+++	+++	+++	+++	++	*K. marxianus* (LC093954)	*K. marxianus*
BUNL-24	+++	+++	-	-	+	+	+	-	NI	*P. kudriavzevii*
BUNL-25	+++	+++	-	-	+	+	+	-	*P. kudriavzevii* (LC093955)	*P. kudriavzevii*
BUNL-26	+++	+++	-	-	+	+	-	-	NI	*P. kudriavzevii*
BUNL-27	+++	+++	-	-	+++	+++	-	-	NI	*C. rhodanensis*
BUNL-28	+++	+++	-	-	+	+	-	-	NI	*P. kudriavzevii*
BUNL-29	+++	+++	+	-	+	+	-	-	NI	*P. kudriavzevii*
BUNL-30	+++	+++	-	-	+	+	-	-	NI	*P. kudriavzevii*
BUNL-31	+++	+++	+++	+++	+	+	-	-	NI	*P. kudriavzevii*
DMKU 3-1042	+++	+++	+++	++	+++	+++	+++	++	*K. marxianus*	*K. marxianus*

[a]+++, strong growth; ++, medium growth; +, less growth; -, no growth. [b]Identification was performed by nucleotide sequencing of D1/D2 domain. NI: Not identified. [c]Identification was performed by the whole-cell MALDI-TOF/MS analysis. [d]Numbers in parentheses are GenBank accession numbers.

Table 2. Ethanol production of the isolated strains in YP + 16% D medium at 40°C.

Strains	Ethanol concentration (% (w/v))		
	12 h	24 h	48 h
BUNL-1	3.23 ± 0.09a	5.55 ± 0.02*	5.32 ± 0.46*
BUNL-2	2.64 ± 0.13	6.34 ± 0.11*	5.22 ± 0.09*
BUNL-3	3.04 ± 0.18	5.54 ± 0.04*	5.24 ± 0.20*
BUNL-4	3.17 ± 0.21	5.91 ± 0.02*	5.19 ± 0.17*
BUNL-5	3.27 ± 0.03	5.75 ± 0.23*	5.54 ± 0.59*
BUNL-6	2.84 ± 0.10	5.36 ± 0.19*	6.29 ± 0.00**
BUNL-7	2.85 ± 0.01	5.25 ± 0.27*	5.50 ± 0.32*
BUNL-8	3.29 ± 0.48	5.83 ± 0.20*	6.11 ± 0.06**
BUNL-9	2.84 ± 0.09	5.45 ± 0.08*	3.23 ± 0.08
BUNL-10	3.42 ± 0.14	5.72 ± 0.06*	4.89 ± 0.39*
BUNL-12	1.81 ± 0.03	3.83 ± 0.22	4.20 ± 0.12
BUNL-13	3.59 ± 0.14	5.62 ± 0.19*	5.58 ± 0.39*
BUNL-14	6.56 ± 0.50**	5.39 ± 0.09*	5.08 ± 0.05*
BUNL-15	4.94 ± 0.26*	5.42 ± 0.36*	5.08 ± 0.05*
BUNL-16	4.76 ± 1.36*	5.76 ± 0.19*	5.83 ± 0.86*
BUNL-17	5.51 ± 0.02*	7.24 ± 1.86*	5.18 ± 0.16*
BUNL-18	3.51 ± 0.30	5.55 ± 0.07*	5.70 ± 0.15*
BUNL-19	3.86 ± 0.29	5.76 ± 0.17*	5.79 ± 1.00*
BUNL-20	3.92 ± 0.54	5.83 ± 0.27*	6.12 ± 0.23**
BUNL-21	5.74 ± 0.24*	6.87 ± 0.46**	5.05 ± 0.02*
BUNL-22	3.72 ± 0.05	5.81 ± 0.00*	5.25 ± 0.09*
BUNL-23	5.34 ± 0.89**	6.04 ± 0.60*	5.09 ± 0.01*
BUNL-24	2.21 ± 0.58	5.69 ± 0.70*	4.36 ± 0.08
BUNL-25	3.40 ± 0.17	6.38 ± 1.87*	5.69 ± 0.57*
BUNL-26	0.9 0± 0.15	5.35 ± 1.43*	5.28 ± 0.17*
BUNL-27	3.32 ± 0.06	7.79 ± 2.00*	5.95 ± 0.46*
BUNL-28	0.01 ± 0.00	0.09 ± 0.04	0.11 ± 0.02
BUNL-29	0.92 ± 0.04	4.14 ± 0.70	6.95 ± 0.31**
BUNL-30	0.62 ± 0.03	3.30 ± 0.00	5.10 ± 0.01*
BUNL-31	0.54 ± 0.00	3.70 ± 0.00	5.98 ± 0.00**
BUNL-32	0.27 ± 0.01	4.05 ± 0.00	5.72 ± 0.00*
DMKU 3-1042	5.10 ± 0.50	5.72 ± 0.45	5.28 ± 0.25

[a]± Standard deviation of values from experiments in triplicate.*Values equivalent to that of DMKU3-1042 as a control within the same time point (t-test; $P>0.05$). **Values significantly higher than that of DMKU 3-1042 within the same time point (t-test; $P<0.05$).

Table 3. Ethanol production of the selected strains in YP + 16% S medium at 40°C.

Strains	Ethanol concentration (% (w/v))		
	12 h	24 h	48 h
BUNL-14	5.03 ± 0.61[a]	5.12 ± 0.05	4.37 ± 0.17
BUNL-15	4.88 ± 0.26*	5.17 ± 0.02	4.34 ± 0.15
BUNL-17	4.70 ± 0.10*	6.71 ± 0.44**	4.99 ± 0.39*
BUNL-21	4.82 ± 0.01*	5.16 ± 0.01	4.75 ± 0.25*
BUNL-23	5.27 ± 0.01**	5.72 ± 0.11*	4.45 ± 0.20
DMKU 3-1042	4.71 ± 0.51	5.65 ± 0.06	4.94 ± 0.12

[a]± Standard deviation of values from experiments in triplicate. *Values equivalent to that of DMKU 3-1042 as a control within the same time point (t-test; $P>0.05$). **Values significantly higher than that of DMKU 3-1042 within the same time point (t-test; $P<0.05$).

Table 4. Ethanol production of the selected strains in 16% sugarcane medium at 40°C.

Strains	Ethanol concentration (% (w/v))		
	12 h	24 h	48 h
BUNL-14	2.89 ± 0.39**[a]	5.68 ± 0.01*	6.52 ± 0.19*
BUNL-15	2.61 ± 0.02**	5.39 ± 0.03*	6.99 ± 0.48*
BUNL-17	2.26 ± 0.08**	5.19 ± 0.49*	7.31 ± 0.68*
BUNL-21	2.68 ± 0.06**	5.39 ± 0.17*	7.31 ± 1.05*
BUNL-23	2.65 ± 0.08**	5.59 ± 0.29*	6.46 ± 0.03
DMKU 3-1042	2.03 ± 0.02	5.15 ± 0.48	6.64 ± 0.04

[a]± Standard deviation of values from experiments in triplicate. *Values equivalent to that of DMKU 3-1042 as a control within the same time point (t-test; $P>0.05$). **Values significantly higher than that of DMKU 3-1042 within the same time point (t-test; $P<0.05$).

Table 5. Ethanol production of the selected strains in 16% molasses medium at 40°C.

Strains	Ethanol concentration (% (w/v))		
	12 h	24 h	48 h
BUNL-14	0.83 ± 0.46**[a]	3.48 ± 0.48**	3.45 ± 0.14
BUNL-15	0.92 ± 0.02**	3.04 ± 0.11**	3.50 ± 0.03
BUNL-17	0.85 ± 0.04**	2.94 ± 0.40**	3.57 ± 0.04*
BUNL-21	0.99 ± 0.18**	3.31 ± 0.26**	4.02 ± 0.24*
BUNL-23	1.15 ± 0.33**	3.35 ± 0.60**	3.79 ± 0.12*
DMKU 3-1042	0.00 ± 0.00	1.58 ± 0.15	5.22 ± 0.95

[a]± Standard deviation of values from experiments in triplicate. *Values equivalent to that of DMKU 3-1042 as a control within the same time point (t-test; $P>0.05$). **Values significantly higher than that of DMKU 3-1042 within the same time point (t-test; $P<0.05$).

Table 6. Ethanol production of the selected strains in YP + 16% D medium at 45°C.

Strains	Ethanol concentration (% (w/v))		
	12 h	24 h	48 h
BUNL-14	4.14 ± 0.47[a]	4.60 ± 0.04	4.41 ± 0.03*
BUNL-15	4.18 ± 0.25**	4.65 ± 0.03*	4.52 ± 0.34*
BUNL-17	4.00 ± 0.09**	5.39 ± 0.08*	4.80 ± 0.44*
BUNL-21	4.35 ± 0.11**	4.80 ± 0.08*	4.47 ± 0.17*
BUNL-23	4.21 ± 0.25**	4.71 ± 0.04*	4.63 ± 0.11*
DMKU 3-1042	3.55 ± 0.26	5.20 ± 0.33	4.61 ± 0.14

[a]± Standard deviation of values from experiments in triplicate. *Values equivalent to that of DMKU 3-1042 as a control within the same time point (t-test; $P>0.05$). **Values significantly higher than that of DMKU 3-1042 within the same time point (t-test; $P<0.05$).

and 21 produced ethanol equivalent to that of DMKU 3-1042 even at 48 h. In the 16% molasses medium, all of the strains produced higher levels of ethanol than those of DMKU 3-1042 at 12 and 24 h, and BUNL-17, 21, and 23 produced ethanol equivalent to that of DMKU 3-1042 at 48 h.

Further fermentation experiments were performed at 45°C with cells being grown in YP + 16% D medium under a shaking condition as shown in Table 6. All of the strains except for BUNL-14 produced higher levels of ethanol than that of DMKU 3-1042 at 12 h. At 24 and 48 h, BUNL-17 showed the highest values of ethanol production though statistically most strains produced ethanol at the levels equivalent to those of DMKU 3-1042. These data suggest that BUNL-17 is the most efficient strain among the isolated strains in YP + 16% D medium at high temperatures.

The five selected strains were identified as *K. marxianus* (see below). They were found to be more efficient ethanol producers than DMKU 3-1042 in glucose,

Table 7. Summary of ethanol production of the selected strains under various conditions.

Strains/ Conditions	Time of fermentation	Sugar consumption (% (w/v))	Ethanol (% (w/v))	Ethanol yield (g/g)	Productivity (g/l/h)
YP + 16% S medium at 40°C					
BUNL-14	24	16	5.12 ± 0.05	0.32 ± 0.00	2.13 ± 0.02
BUNL-15	24	16	5.17 ± 0.02	0.32 ± 0.00	2.15 ± 0.01
BUNL-17	24	16	6.71 ± 0.44**	0.42 ± 0.03**	2.80 ± 0.13**
BUNL-21	24	16	5.16 ± 0.01	0.32 ± 0.00	2.14 ± 0.01
BUNL-23	24	16	5.72 ± 0.11*	0.36 ± 0.01*	2.38 ± 0.05*
DMKU 3-1042	24	16	5.65 ± 0.06	0.35 ± 0.01	2.35 ± 0.25
16% sugarcane medium at 40°C					
BUNL-14	48	16	6.52 ± 0.19*	0.41 ± 0.01*	1.36 ± 0.03*
BUNL-15	48	16	6.99 ± 0.48*	0.44 ± 0.02*	1.46 ± 0.07*
BUNL-17	48	16	7.31 ± 0.68*	0.46 ± 0.03*	1.52 ± 0.10*
BUNL-21	48	16	7.31 ± 1.05*	0.46 ± 0.05*	1.52 ± 0.16*
BUNL-23	48	16	6.46 ± 0.03	0.41 ± 0.00*	1.35 ± 0.01
DMKU 3-1042	48	16	6.64 ± 0.04	0.42 ± 0.01	1.38 ± 0.01
16% molasses medium at 40°C					
BUNL-14	48	8.2	3.48 ± 0.48	0.42 ± 0.01*	0.73 ± 0.10
BUNL-15	48	8.4	3.5 ± 0.03	0.42 ± 0.00*	0.73 ± 0.01
BUNL-17	48	8.0	3.57 ± 0.04*	0.45 ± 0.00*	0.74 ± 0.01
BUNL-21	48	8.9	4.02 ± 0.24*	0.45 ± 0.02*	0.84 ± 0.05*
BUNL-23	48	10	3.79 ± 0.12*	0.38 ± 0.01*	0.79 ± 0.03*
DMKU 3-1042	48	12.5	5.22 ± 0.95	0.42 ± 0.06	1.09 ± 0.20
YP + 16% D medium at 45°C					
BUNL-14	24	16	4.60 ± 0.04	0.29 ± 0.00*	1.92 ± 0.02
BUNL-15	24	16	4.65 ± 0.03*	0.29 ± 0.00*	1.94 ± 0.01*
BUNL-17	24	16	5.39 ± 0.08*	0.34 ± 0.01*	2.25 ± 0.03*
BUNL-21	24	16	4.80 ± 0.08*	0.30 ± 0.01*	2.00 ± 0.03*
BUNL-23	24	16	4.71 ± 0.04*	0.29 ± 0.00*	1.96 ± 0.02*
DMKU 3-1042	24	16	5.20 ± 0.33	0.33 ± 0.02	2.17 ± 0.14

*Values equivalent to that of DMKU 3-1042 as a control within the same time point (t-test; $P>0.05$). **Values significantly higher than that of DMKU3-1042 within the same time point (t-test; $P<0.05$).

sucrose, sugarcane, and molasses media at least in the early incubation period (12 and/or 24 h) at 40°C. They showed different capabilities of ethanol fermentation in different carbon sources: 5.12 to 6.71% at 24 h in YP + 16% S medium (Table 3), 6.46 to 7.31% (w/v) at 48 h in 16% sugarcane medium (Table 4) and 3.45 to 4.02% (w/v) at 48 h in 16% molasses medium (Table 5). BUNL-17 exhibited the highest values of ethanol production among the five strains in YP + 16% D medium at 45°C after 24 h (5.39% (w/v) at 24 h, 4.80% (w/v) at 48 h) (Table 6), and its values of ethanol productivity were greater than those of DMKU 3-1042 in YP + 16% S medium at 24 h and in 16% sugarcane medium at 48 h and in YP + 16% D medium at 45°C at 48 h (Table 7). Notably, BUNL-27, *Cyberlindnera rhodanensis,* showed the highest value of ethanol production (7.79% (w/v) at 24 h) in YP + 16% D medium at 40°C (Table 2), and DMKU 3-1042 showed the highest value of ethanol productivity in 16% molasses medium at 48 h (Table 7). These findings allow us to speculate that there are efficient strains specific for each biomass.

Identification of some of the isolated strains by nucleotide sequencing

Analysis of the D1/D2 domain of the large subunit rRNA gene was performed for 16 strains, and they were identified as *Pichia kudriavzevii*, *C. rhodanensis* and *K. marxianus* as shown in Table S1 and Table 1. Consistently, phylogenetic tree based on sequences of the D1/D2 domain in the large-subunit rDNA gene demonstrated that eight strains of BUNL-1, BUNL-2, BUNL-4, BUNL-6, BUNL-8, BUNL-10, BUNL-11, and BUNL-25 were located in the same position as *P. kudriavzevii* (Figure 1). Strain BUNL-12 was located in

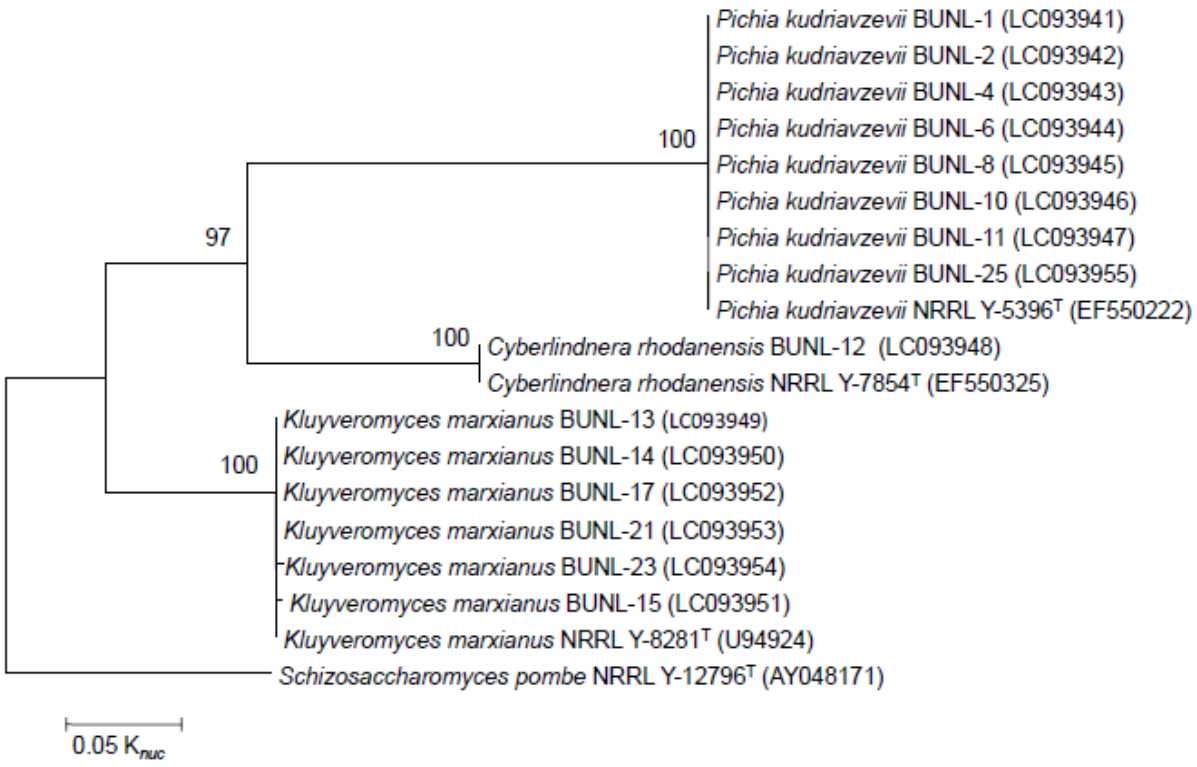

Figure 1. Phylogenetic tree based on the sequences of the D1/D2 region of the LSU rRNA gene, showing positions of the isolated strains with respect to type strain of each species. The phylogenetic tree was constructed as described in Materials and Methods. Numbers indicate percentages of bootstrap sampling, derived from 1,000 samples. The numbers in parentheses are GenBank accession numbers. *Schizosaccharomyces pombe* NRRL Y-12796^T was outgroup in the analysis. Bar represents 0.05 K_{nuc} distance.

the same position as *C. rhodanensis* and six strains of BUNL-13, BUNL-14, BUNL-15, BUNL-17, BUNL-21, and BUNL-23) were located in the same position as *K. marxianus*. Colony morphologies of all other unidentified strains except for BUNL-27 were similar to those of strains identified as *P. kudriavzevii,* which are dull to occasionally almost powdery, light- cream colored and entirely undulate margin. The colony morphology of BUNL-27 was small, smooth, glistening, white colored and entirely filamentous margin, which was similar to that of BUNL-12.

Characterization of isolated strains by whole-cell MALDI-TOF/MS analysis

Whole-cell MALDI-TOF/MS analysis was introduced as a high-throughput identification procedure for identifying known/novel species of bacteria without 16S rRNA gene sequencing (Tani et al., 2015), and it has also been used for characterization of bacteria in ballast water (Emami et al., 2012). this procedure was thus applied for identification of yeast strains isolated in this study, and the resultant spectra data were subjected to clustering analysis using BioTyper software (Figure 2). The analysis revealed that there were three clusters with each cluster including *P. kudriavzevii*, *C. rhodanensis* and *K. marxianus* strains that were identified by analysis of the D1/D2 domain of the large subunit rRNA gene (Table S1). This clustering allowed us to identify all of the isolated strains as shown in Table 1. All of the identifications were consistent with colony morphologies described earlier. Taken together, it is concluded that three yeast species were isolated from various samples in Laos by the screening method applied in this study.

DISCUSSION

The acquisition of a thermotolerant fermenting microbe suitable for individual biomass is indispensable for high-temperature ethanol fermentation or for fermentation under temperature-uncontrolled conditions (Murata et al., 2015). A simple and fast procedure for screening of such microbes is thus desired. In this study, two procedures were tested: (1) an enrichment procedure at relatively high temperatures and (2) whole-cell MALDI-TOF/MS analysis for quick identification of species. This, as far as known, is the first large-scale screening of thermotolerant yeasts in Laos as one of the tropical countries.

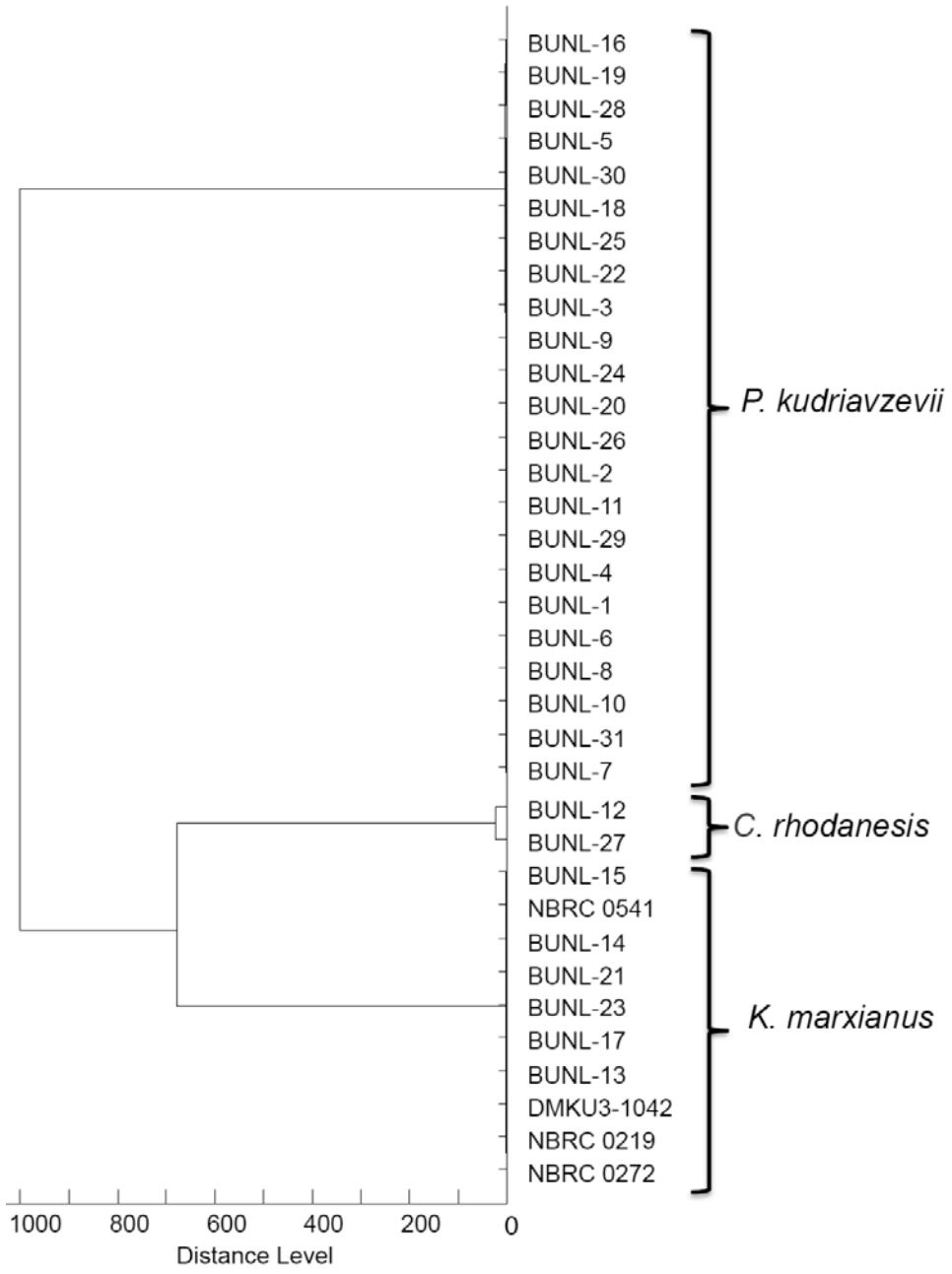

Figure 2. BioTyper-created dendrogram based on spectral data of all of the isolated strains by whole-cell MALDI-TOF/MS analysis. Whole-cell MALDI-TOF/MS and BioTyper analyses were performed as described in Methods. DMKU 3-1042 and NBRC 0219 were thermotolerant *K. marxianus* strains, and NBRC 0541 and NBRC 0272 were less- thermotolerant *K. marxianus* strains (Lertwattanasakul et al., 2015).

In respect to fermentation capabilities of isolates reported previously as thermotolerant yeasts, it has been shown that a *Saccharomyces diastaticus* strain produced 6.4% (w/v) ethanol from 15% glucose at 40°C (D'Amore et al., 1989) and a *S. cerevisiae* R-8 produced 4.8% (w/v) ethanol from 10% glucose at 37°C (Brooks, 2008). In *K. marxianus,* a strain DMKU 3-1042 performed the production of ethanol over 6% (w/v) from 22% sugarcane juice at 40°C (Limtong et al., 2007) and a strain TISTR 5925 produced about 4.5% (w/v) from 10% glucose at 42°C (Apiwatanapiwat et al., 2013). Fermentation applications revealed that a strain L.G. produced 3.8% (w/v) ethanol from hydrolyzed 10% Solka-floc (potential glucose content in Solka-floc is 94.5% dry weight basis)

at 42°C by simultaneous saccharification and fermentation (SSF) procedure (Ballesteros et al., 1991) and a strain DBTIOC-35 produced 2.9 (w/v) and 6.2% (w/v) from 10 and 20% biomass (acid pretreated wheat straw), respectively, at 42°C by SSF (Saini et al., 2015). Comparison with these previous achievements reveal that strains isolated in this study, especially BUNL-17 and BUNL-27, have potentials in ethanol fermentation, equivalent to or more than those of strains isolated previously. In addition, fermentation ability has been examined preliminarily with rice hydrolysate as reported previously (Murata et al., 2015). BUNL-17 and DMKU 3-1042 produced over 3% (w/v) and 2% (w/v), respectively, at 40°C in rice hydrolysate medium (equivalent to 10% glucose) supplemented with 0.05% $(NH_4)_2SO_4$, 0.5% KH_2PO_4 and 0.15% $MgSO_4 \cdot 7H_2O$ under a shaking condition at 120 rpm.

As described earlier, whole-cell MALDI-TOF/MS analysis allowed us to divide the isolates to three groups. Strain identification by the two types of analyses matched exactly. It appeared that the thermotolerance and positions of each isolate in the dendrogram did not correlate. It is known that the peaks detected in whole-cell MALDI-TOF/MS analysis are mostly from ribosomal proteins. Thus, the m/z pattern of ribosomal proteins may not be a direct indicator of thermotolerance. Suggestive evidence that divergent copies of ribosomal operons as paralogues in an archaea, *Haloarcula marismortui*, may improve fitness at high and low temperatures (Lo´pez-Lo´pez et al., 2007) allowed us to compare the patterns of MALDI-TOF/MS peaks between thermotolerant and less-thermotolerant *K. marxianus* species reported previously (Lertwattanasakul et al., 2015). However, no significant difference in the patterns was observed between them (data not shown). On the other hand, only three species were obtained in this study. The small variety of species might be due to the application of the enrichment culture in the screening process, in which the major population of microbes may prevent growth of the minor population of microbes. It may thus be necessary to apply a non-enrichment culture for the acquisition of various species.

Conflict of Interests

The authors have not declared any conflict of interests.

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REFERENCES

Altschul SF, Gish W, Miller W, Meyers EW, Lipman DJ (1990). Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Apiwatanapiwat W, Rugthaworn P, Valithanomsat P, Thanapase W, Kosugi A, Arai T, Mori Y, Murata Y (2013) Ethanol production at high temperature from cassava pulp by a newly isolated *Kluyveromyces marxianus* strain, TISTR 5925. AIMS Energy.1:3-16.

Ballesteros I, Negro MJ, Oliva JM, Cabanas A, Manzanares P, Ballesteros M (2006). Ethanol production from steam-explosion pretreated wheat straw. Appl. Biochem. Biotechnol. 130:496-508.

Banat IM, Nigam P, Marchant R (1992). Isolation of thermotolerant, fermentative yeasts growing at 52°C and producing ethanol at 45°C and 50°C. World J. Microbiol. Biotechnol. 8:259-263.

Brooks AA (2008). Ethanol production potential of local yeast strains isolated from ripe banana peels. Afr. J. Biotechnol. 7:3749-3752.

Emami K, Askari V, Ullrich M, Mohinudeen K, Anil CA, Khandeparker L, Burgess GJ, Mesbahi E (2012). Characterization of Bacteria in Ballast Water Using MALDI-TOF Mass Spectrometry. PLoS One 7:e38515.

Felsenstein J (1985). Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783-791.

Green MR, Sambrook J (2012). Molecular Cloning: A Laboratory Manual, fourth ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Kida K, Kume K, Norimura S, Sonoda Y (1992). Repeated-batch fermentation process using a thermotolerant flocculating yeast constructed by protoplast fusion. J. Ferment. Bioeng.74:169-173.

Kimura M (1980). A simple method for estimating evolutionary rate of base substitutions though comparative studies of nucleotide sequence. J. Mol. Evol. 16:111-120.

Lertwattanasakul N, Kosaka T, Hosoyama A, Suzuki Y, Rodrussamee N, Matsutani M, Murata M, Fujimoto N, Suprayogi, Tsuchikane K, Limtong S, Fujita N, Yamada M (2015). Genetic basis of the highly efficient yeast *Kluyveromyces marxianus*: complete genome sequence and transcriptome analyses. Biotechnol. Biofuels 8:47.

Lertwattanasakul N, Rodrussamee N, Suprayogi, Limtong S, Thanonkeo P, Kosaka T, Yamada M (2011). Utilization capability of sucrose, raffinose and inulin and its less- sensitiveness to glucose repression in thermotolerant yeast *Kluyveromyces marxianus* DMKU 3-1042. AMB Express 1:20.

Limtong S, Sringiew C, Yongmanitchai W (2007). Production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *Kluyveromyces marxianus*. Bioresour. Technol. 98: 3367-3374.

Lo´pez-Lo´pez A, Benlloch S, Bonfa M, Rodr´ıguez-Valera F, Mira A (2007). Intragenomic 16S rDNA Divergence in Haloarcula marismortui Is an Adaptation to Different Temperatures. J. Mol. Evol. 65:687-696.

Morimura S, Zhong YL, Kida K (1997). Ethanol production by repeated-batch fermentation at high concentration in a molasses medium containing a high concentration of total sugar by a thermotolerant flocculating yeast with improved salt-tolerance. J. Ferment. Bioeng. 83:271-274.

Murata M, Nitiyon S, Lertwattanasakul N, Sootsuwan K, Kosaka T, Thanonkeo P, Limtong S, Yamada M (2015). High-temperature fermentation technology for low-cost bioethanol. J. Jpn. Inst. Energy, 94:1154-1162..

O'Donnell K (1993). Fusarium and its near relatives. In: Reynolds DR, and Taylor JW (Eds.) The Fungal Holomorph: Mitotic, Meiotic and Pleomorphic Speciation in Fungal Systematics. CAB International, Wallingford, pp. 225-233.

Rees J (2014). The Renewable Fuel Standard: Issues for 2014 and Beyond, June 2014 Report of Congressional Budget Office, USA.

Rodrussamee N, Lertwattanasakul N, Hirata K, Suprayogi, Limtong S, Kosaka T, Yamada M (2011). Growth and ethanol fermentation ability on hexose and pentose sugars and glucose effect under various conditions in thermotolerant yeast *Kluyveromyces marxianus*. Appl. Microbiol. Biotechnol. 90:1573-1586.

Saini JK, Agrawal R, Satlewal A, Saini R, Gupta R, Mathur A, Tuli D (2015). Second generation bioethanol production at high gravity of pilot-scale pretreated wheat straw employing newly isolated thermotolerant yeast *Kluyveromyces marxianus* DBTIOC-35. RSC

Adv. 5:37485-37494.
Saitou N, Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406-425.
Sree NK, Sridhar M, Suresh K, Banat IM, Rao LV (2000). Isolation of thermotolerant, osmotolerant, flocculating, *Saccharomyces cerevisiae* for ethanol production. Bioresour. Technol. 72:43-46.
Talebnia F, Karakashev D, Angelidaki I (2010). Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation. Bioresour. Technol. 101:4744-4753.
Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30:2725-2729.
Tani A, Sahin N, Fujitani Y, Kato A, Sato K (2015). *Methylobacterium* species promoting rice and barley growth and interaction specificity revealed with whole-cell matrix-assisted laser desorption / ionization-time-of-flight mass spectrometry (MALDI-TOF/MS) analysis. PLoS One 10:e0129509.
Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic. Acid Res. 24:4876-4882.
Ueno R, Urano N, Kimura S (2001). Characterization of thermotolerant, fermentative yeasts from hot spring drainage. Fish. Sci. 67:138-145.
Yuangsaard N, Yongmanitchai W, Yamada M, Limtong S (2013). Selection and characterization of a newly isolated thermotolerant *Pichia kudriavzevii* strain for ethanol production at high temperature from cassava starch hydrolysate. Antonie Van Leeuwenhoek 103:577-588.

Table S1. Identification of 17 isolated strains by nucleotide sequencing.

Strain	Accession number	Closest species with accession number of type species	Nucleotide identity in D1/D2 domain		E value	Nucleotide different in D1/D2 domain			Result of identification
			Identical nucleotides /total nucleotides	Identity (%)		Number of Gap	nucleotide substitution		
							no.	%	
BUNL-1	LC093941	*Pichia kudriavzevii* (EF550222)	525/527	99.62	0.00	2	0	0.00	*Pichia kudriavzevii*
BUNL-2	LC093942	*Pichia kudriavzevii* (EF550222)	509/510	98.07	0.00	1	0	0.00	*Pichia kudriavzevii*
BUNL-4	LC093943	*Pichia kudriavzevii* (EF550222)	511/514	99.42	0.00	2	1	0.19	*Pichia kudriavzevii*
BUNL-6	LC093944	*Pichia kudriavzevii* (EF550222)	558/558	100.0	0.00	0	0	0.00	*Pichia kudriavzevii*
BUNL-8	LC093945	*Pichia kudriavzevii* (EF550222)	535/538	100.0	0.00	3	0	0.00	*Pichia kudriavzevii*
BUNL-10	LC093946	*Pichia kudriavzevii* (EF550222)	549/552	99.46	0.00	2	1	0.18	*Pichia kudriavzevii*
BUNL-11	LC093947	*Pichia kudriavzevii* (EF550222)	551/552	99.82	0.00	1	0	0.00	*Pichia kudriavzevii*
BUNL-12	LC093948	*Cyberlindnera rhodanensis* (EF550325)	578/579	99.83	0.00	0	1	0.17	*Cyberlindnera rhodanensis*
BUNL-13	LC093949	*Kluyveromyces marxianus* (U94924)	523/525	99.62	0.00	0	0	0.00	*Kluyveromyces marxianus*
BUNL-14	LC093950	*Kluyveromyces marxianus* (U94924)	503/503	100.0	0.00	0	0	0.00	*Kluyveromyces marxianus*
BUNL-15	LC093951	*Kluyveromyces marxianus* (U94924)	496/498	99.60	0.00	1	1	0.20	*Kluyveromyces marxianus*
BUNL-17	LC093952	*Kluyveromyces marxianus* (U94924)	524/525	99.81	0.00	1	0	0.00	*Kluyveromyces marxianus*
BUNL-21	LC093953	*Kluyveromyces marxianus* (U94924)	503/503	100.0	0.00	0	0	0.00	*Kluyveromyces marxianus*
BUNL-23	LC093954	*Kluyveromyces marxianus* (U94924)	499/501	99.60	0.00	1	0	0.00	*Kluyveromyces marxianus*
BUNL-25	LC093955	*Pichia kudriavzevii* (EF550222)	551/552	99.82	0.00	1	0	0.00	*Pichia kudriavzevii*