Agriculture and Natural Resources 52 (2018) 511–518

Contents lists available at ScienceDirect

Agriculture and Natural Resources

journal homepage: http://www.journals.elsevier.com/agriculture-and-natural-resources/

Original Article

High temperature alcoholic fermentation by new thermotolerant yeast strains *Pichia kudriavzevii* isolated from sugarcane field soil

Pongsanat Pongcharoen,[a, b, *] Jariya Chawneua,[a, 1] Wittaya Tawong[a, b, 1]

[a] Department of Agricultural Science, Faculty of Agriculture, Natural Resources and Environment, Naresuan University, Phitsanulok, Thailand
[b] Center of Excellence in Research in Agricultural Biotechnology, Naresuan University, Phitsanulok, Thailand

ARTICLE INFO

Article history:
Received 2 March 2018
Accepted 27 May 2018
Available online 23 November 2018

Keywords:
Ethanol production
Ethanol tolerant
Pichia kudriavzevii
Sugarcane field
Thermotolerant yeast

ABSTRACT

The thermotolerant and ethanogenic yeasts are an important factor in numerous ethanol industrial applications. In this study, the potential of new isolates of thermotolerant, ethanol-producing yeasts was successfully demonstrated. In total, 60 yeast isolates were obtained from soil sugarcane fields in Uttaradit, Kamphang Phet, Chai Nat, Sukhothai, Nakhon Sawan and Phitsanulok provinces, Thailand and subjected to characterization of thermotolerance using an enrichment technique with 4% (volume per volume) ethanol. The growth performance and fermentation activity under stress conditions were compared with that of Thai industrial *Saccharomyces cerevisiae* TISTR 5606. Interestingly, the results showed that 30 isolates grew at high temperatures (up to 45 °C). Three isolates (NUNS–4, NUNS–5 and NUNS–6) could tolerate those conditions on agar composed of yeast extract, peptone and glucose containing 13% (v/v) ethanol. Furthermore, gas chromatography analysis to determine the ethanol concentration revealed the three new isolates produced higher amounts of ethanol than *S. cerevisiae* TISTR 5606 at fermentation temperatures of 40 °C and 45 °C ($p < 0.05$) when utilizing glucose as the carbon source. The isolate NUNS–4 had the highest ethanol concentrations of 88.60 ± 0.75 g/L and 54.30 ± 0.97 g/L at 40 °C and 45 °C, respectively. Furthermore, phylogenetic analysis based on the D1/D2 domain of 26S rDNA showed that the new isolates were identified as *Pichia kudriavzevii*. Consequently, thermo-tolerant and ethanol-tolerant *P. kudriavzevii* NUNS–4, NUNS–5 and NUNS–6 are possible candidates as strains for commercial-scale ethanol production when using glucose as the fermentation substrate.

Introduction

Large amounts of fossil-derived energy and chemicals are consumed globally each year (Auesukaree et al., 2012). Moreover, massive combustion of petroleum oil has also increased the emission of CO_2 resulting in the greenhouse effect and subsequent global warming (Wu et al., 2016). On the other hand, alternative energy sources such as water, wind, nuclear fission/fusion, solar, geothermal or biomass have received much attention not only for protecting the environment, but also for meeting different energy market demands (Wu et al., 2016). Bioethanol is one such renewable energy source whose production using microbial fermentation is developing to replace gasoline (Dhaliwal et al., 2011). Of all microorganisms, yeasts, especially *Saccharomyces* spp., are the most common ethanol producers and are widely used in the ethanol fuel industry (Edgardo et al., 2008). From the thermodynamic perspective, high-temperature ethanol fermentation provides several advantages such as reduced risks of contamination by undesirable microorganisms, reduced cooling costs for bioreactors and increased ethanol productivity (Auesukaree et al., 2012; Apiwatanapiwat et al., 2013; Siedlarz et al., 2016). Due to their tolerance to high ethanol concentrations and toxic substances formed during the fermentation process, yeasts have proven to be more practical for ethanol production than bacteria. Opportunities to overcome the current limitations and challenges for industrial fermentation are resulting in increased explorations of new thermotolerant, ethanol-producing yeasts that are capable of growth and ethanol production at high temperatures.

Yeast species are widely distributed in nature with divergent ecosystems and many researchers have been trying to isolate these

* Corresponding author. Department of Agricultural Science, Faculty of Agriculture, Natural Resources and Environment, Naresuan University, Phitsanulok, Thailand.
E-mail address: pongsanatp@nu.ac.th (P. Pongcharoen).
[1] Equal contribution.

https://doi.org/10.1016/j.anres.2018.11.017

organisms from different ecological niches such as soil in sugarcane, cassava and pineapple plantations (Kaewkrajay et al., 2014; Pongcharoen and Kawano-Kawada, 2018), rotten fruit (Chamnipa et al., 2017) or naturally fermented sources (Talukder et al., 2016) as well as clay soil (Hesham and Mohamed, 2011). Several previous studies have shown that several yeast species isolated from natural habitats have been classified as industrial-thermotolerant yeasts such as *S. cerevisiae* KKU–VN8, KKU–VN20 and KKU–VN27, *Pichia kudriavzevii* KKU–TH33 and KKU–TH43 (Techaparin et al., 2017), *Kluyveromyces marxianus* TISTR 5925 (Apiwatanapiwat et al., 2013) and *P. kudriavzevii* KVMP10 (Koutinas et al., 2016). The sugarcane field, one of the most abundant agricultural ecosystems in Thailand, is promising as one of the best microbial communities offering niche preferences for ethanol-producing yeasts (Buddiwong et al., 2014). Soil receives all or most of its nutrients from external sources such as plants and animals, which are important for yeast utilization; therefore, soil is the true habitat for a number of species of yeasts (Limtong et al., 2009). As expected, natural ecosystems are a major source of useful microorganisms for biorefinery production (Buddiwong et al., 2014). The most successful procedure reported to date involves collecting naturally occurring isolates which exhibit their ability to grow at high temperature as well as producing ethanol (Banat et al., 1998). There is substantial interest in exploring these natural isolates because they have many advantageous characteristics and specific properties which are unavailable in the laboratory strains. This is especially relevant for Thailand, a tropical area, where daytime temperatures are usually higher than 35 °C and the species diversity of yeast is rich in the natural environment (Sree et al., 2000). Thus, it is an interesting research area for the selection of some novel yeasts and also to find their useful functions for industrial applications.

The objectives of the present work were to characterize useful thermotolerant and ethanol-tolerant yeasts isolated from soil sugarcane fields in Thailand and to examine their potential for ethanol production at high temperatures. Among several new isolates in this study, three thermotolerant yeast isolates identified as *P. kudriavzevii* exhibited a unique characteristic in growth performance under stress conditions of high temperature and ethanol concentration. Finally, the ethanol production capabilities of these three new *P. kudriavzevii* isolates under high temperature were also investigated.

Materials and methods

Sample collection and isolation

Soil samples from sugarcane fields were collected from Uttaradit (UD), Kamphaeng Phet (KP), Chai Nat (CN), Sukhothai (ST), Nakhon Sawan (NS) and Phitsanulok (PHS) provinces, Thailand (Table 1). Isolation was carried out using an enrichment technique with yeast peptone dextrose (YPD) broth (1% yeast extract, 2% peptone and 2% glucose) supplemented with 4% (volume per volume; v/v) ethanol, 0.025% sodium propionate (P1880; Sigma; Oakville, Canada) and 0.02% chloramphenicol (C0378, Sigma; St. Louis, USA) (Yuangsaard et al., 2013). Two grams of a soil sample were added to 50 mL of YPD broth containing antibiotic and 4% (v/v) ethanol concentration and incubated at room temperature for 24 hr. Then, the enrichment culture was streaked on a YPD agar plate and incubated at 37 °C until a single colony appeared. All pure cultures were collected and maintained on YPD agar at 4 °C for short-term storage. For long-term storage, a single colony of each isolate was suspended in YPD broth supplemented with 25% glycerol and maintained at −80 °C. For the reference species, *S. cerevisiae* TISTR 5606 (an industrial ethanol-producing strain) was purchased from the Thailand Institute of Scientific and Technological Research, Bangkok, Thailand.

Examining thermotolerance and ethanol-tolerance of yeasts

Yeast isolates were pre-cultivated in YPD broth at 30 °C, then inoculated into fresh YPD broth until growth reached the early stationary phase (approximately 9–12 hr). The growth performance of each yeast isolate at high temperature (45 °C) was tested using the modified method described by Yuangsaard et al. (2013) and Techaparin et al. (2017). To determine the growth ability with different ethanol-supplemented levels, ethanol was added to YPD agar at 7%, 10%, 13% and 15% (v/v). The growth of yeast was monitored at temperatures of 37 °C and 45 °C. Each experiment was repeated twice.

Fermentation activity test of thermotolerant yeast at high temperature

Initially, yeast isolates were tested for ethanol production at 37 °C, 40 °C, 42 °C and 45 °C in YPD broth. Screening of ethanol fermentation was performed using a Durham fermentation tube with 0.1 mL of active cells inoculated into YPD broth (9 mL) in a test tube (18 mm diameter × 150 mm) containing a Durham tube (Buddiwong et al., 2014) and then statically incubated at different temperatures. The accumulation of CO_2 gas in the Durham tube was monitored every 6 hr. The isolates which contained gas within the Durham tube were selected for further analysis.

To determine the sugar utilization ability of yeast isolates, the modified method described by Buddiwong et al. (2014) was conducted. Briefly, YP broth containing 2% (w/v) glucose, sucrose or xylose as a carbon source was prepared in this study. Each yeast isolate was cultured on YP broth containing different carbon sources using the Durham tube. Then, all cultures tested were maintained at 37 °C. Gas production in the Durham tube was recorded. All experiments were run in triplicate.

Estimation of ethanol production

For quantitative estimation of ethanol production using gas chromatography analyses, yeast cells were aerobically pre-cultivated to the exponential phase in YPD broth at 30 °C, and

Table 1
Abbreviated names of yeast isolates from soil in sugarcane fields and numbers of yeasts at different locations.

Location	Abbreviated name	Number of soil samples	Number of isolates
Uttaradit	NUUD	3	12
Kamphang Phet	NUKP	3	12
Chai Nat	NUCN	3	6
Sukhothai	NUST	3	12
Nakhon Sawan	NUNS	3	6
Phitsanulok	NUPHS	3	12
Total			60

then were inoculated into a 250 mL Erlenmeyer flask containing 100 mL fresh medium of YPD broth containing 16% (w/v) glucose as a carbon source. The initial optical density (OD_{660}) value of cells before further cultivation was 0.1 and incubation occurred at specific temperatures (40 °C and 45 °C) in a shaker incubator at 135 rpm (Yuangsaard et al., 2013). A portion of the fermentation broth was withdrawn from each flask after 24hr, 36hr and 48hr incubation and centrifuged at 16,200×*g* for 10 min at 4 °C. The supernatant was collected and subjected to gas chromatography (GC–14B; Shimadzu; Kyoto, Japan) in order to measure the concentration of ethanol. One-way analysis of variance and Duncan's multiple range tests were used to test for significant ($p < 0.05$) differences in the ethanol production between the isolated and reference isolate using the R program (version 3.2.4; https://www.r-project.org/).

Identification of the yeast isolate

Genomic DNA was extracted from the yeast cells using the lithium acetate DNA extraction method (Lõoke et al., 2011). In order to amplify the D1/D2 domain of the larger subunit (LSU) 26S rDNA region, the primers NL–1 (5′–GCATATCAATAAGCGGAGGAAAAG–3′) and NL–4 (5′–GGTCCGTGTTTCAAGACGG–3′) were used with the PCR conditions as described by Techaparin et al. (2017). All amplification products were purified using a FastGene™ Gel/PCR Extraction Kit (Nippon Genetics; Tokyo, Japan) and directly sequenced using ABI a BigDye Terminator version 3.1 Cycle sequencing kit and automated Prism 3730XL DNA Analyzer (Applied Biosystems; Foster City, CA, USA). After sequencing, the obtained nucleotides were initially examined using the BLAST program (National Center for Biotechnology Information; http://ncbi.nlm.nih.gov/) to search for homologous sequences from other yeast species. Subsequently, the D1/D2 LSU rDNA sequences of new isolates from this work and those sequences of other thermotolerant yeasts retrieved from the database were aligned using the CLUSTAL W software (Thompson et al., 1994), implemented in the UGENE software (Okonechnikov et al., 2012). The phylogenetic trees were constructed using the neighbor-joining (NJ) and maximum likelihood (ML) methods. The ML tree was performed using the GTR + G + I model with the PhyML 3.0 software (Guindon et al., 2010) and each topology was verified using 100 bootstrap replications. The NJ tree was generated from evolutionary distance data calculated using the p distance method with 1000 bootstrap replications implemented in the MEGA7 software (Kumar et al., 2016). In this study, the D1/D2 LSU sequences of the NUNS–4, NUNS–5 and NUNS–6 isolates were deposited in GeneBank as LC371651, LC371652 and LC371653, respectively.

Results

Screening of thermotolerant-ethanol tolerant yeast

In total, 60 yeast isolates were obtained using an enrichment culture YPD broth and groing on YPD agar at 37 °C (Fig. 1A). Among them, 30 isolates could grow at 45 °C, specifically NUCN–1, NUCN–2, NUCN–3, NUCN–4, NUCN–5, NUCN–6, NUNS–4, NUNS–5, NUNS–6, NUST–1, NUST–2, NUST–3, NUUD–1, NUUD–2, NUUD–3, NUUD–4, NUUD–5, NUUD–6, NUUD–7, NUUD–8, NUUD–9, NUUD–10, NUUD–11, NUUD–12, NUKP–1, NUKP–2, NUKP–3, NUKP–4, NUKP–5 and NUKP–6 (Fig. 1B). Nevertheless, six of these 30 isolates (NUCN–1, NUCN–2, NUCN–3, NUCN–4, NUCN–5 and NUCN–6) were less tolerant to 45 °C than other isolates. Considering the definition described by Sree et al. (2000) and Chamnipa et al. (2017), these 30 isolates were categorized as thermotolerant yeasts since they were able to grow at temperature higher than 40 °C.

Moreover, all 30 isolates were able to be grown on YPD agar containing 7% (v/v) ethanol. Of the 30 isolates, 13 isolates (NUNS–4, NUNS–5, NUNS–6, NUST–2, NUST–3, NUUD–1, NUUD–2, NUUD–3, NUUD–4, NUUD–5, NUUD–6, NUUD–7 and NUUD–80 exhibited ability to grow on the YPD agar containing up to 10% (v/v) ethanol as well as the reference strain. However, the others did not have the ability to tolerate concentrations of 10% (v/v) ethanol. Interestingly, three isolates (NUNS–4, NUNS–5 and NUNS–60 grew in YPD agar containing 13% (v/v) ethanol (Fig. 2). Nevertheless, none of the 30 isolates grew at ethanol concentrations of 15% (v/v; data not shown).

Screening of thermotolerant fermentative yeast

For considering the fermentative ability of various strains at high temperatures, 30 isolates selected from the previous experiment were initially determined by monitoring their gas production in Durham test tubes. The results showed that all 30 yeast isolates had fermentation activity with generation of CO_2 at 37 °C, 40 °C and 42 °C within 36 hr incubation using glucose as a carbon source. Interestingly, only isolates of NUNS–4, NUNS–5 and NUNS–6 had gas production at 45 °C (data not shown). Furthermore, all 30 isolates of yeast were grown on YP broth containing different carbon sources (sucrose and xylose) and were incubated at 37 °C. The results showed that all the NUUD, NUKP, NUCN, NUST and NUPHS isolates could utilize sucrose, but not xylose, as their carbon source. However, NUNS–4, NUNS–5 and NUNS–6 could utilize neither sucrose nor xylose (data not shown).

Ethanol production at different temperature by gas chromatography

In this study, ethanol productivity of NUNS–4, NUNS–5, NUNS–6 and *S. cerevisiae* TISTR 5606 were investigated in fermentation using YPD broth with 16% (w/v) glucose at 40 °C and 45 °C (Table 2). After 24hr fermentation at 40 °C, the level of ethanol production of NUNS–4 (58.78 ± 0.51 g/L), NUNS–5 (49.39 ± 1.04 g/L) and NUNS–6 (53.16 ± 0.59 g/L) were significantly higher than TISTR 5606 (34.37 ± 0.74 g/L). Then, maximum ethanol concentrations were found after 48hr incubation at 40 °C by NUNS–4 (88.60 ± 0.75 g/L), NUNS–5 (78.52 ± 0.21 g/L), NUNS–6 (77.97 ± 0.65 g/L) and TISTR 5606 (65.37 ± 0.82 g/L), respectively. When incubated at 45 °C, the isolates, NUNS–4, NUNS–5 and NUNS–6, remained at high ethanol productivity levels compared to the reference strain ($p < 0.05$). The highest ethanol production levels at 45 °C were found with the isolates NUNS–4 (54.30 ± 0.97 g/L), NUNS–5 (37.73 ± 1.46 g/L) and NUNS–6 (44.04 ± 0.92 g/L). The reference could only produce its highest ethanol concentration of 4.067 ± 0.38 g/L at 45 °C after 36 hr incubation.

Identification of selected thermotolerant yeasts

The BLAST results showed that the D1/D2 LSU rDNA sequences obtained in this study were identical (100% identity, E-value = 0.00 and 525 nucleotide coverage) with the D1/D2 LSU rDNA sequence of the type of *P. kudriavzevii* (CBS 12548). Based on the sequence of the D1/D2 domain of the large subunit rDNA, the ML and NJ phylogenetic trees had identical topologies, and thus only the ML tree is presented in Fig. 3. The data revealed that three isolates (NUNS–4, NUNS–5 and NUNS–6) were grouped together with the type strain of *P. kudriavzevii* (NRRL Y–5396) in the *P. kudriavzevii* clade with robust supported values from the ML (100) and NJ (100) methods. Therefore, the isolates Thai NUNS–4, NUNS–5 and NUNS–6 were identified as *P. kudriavzevii*.

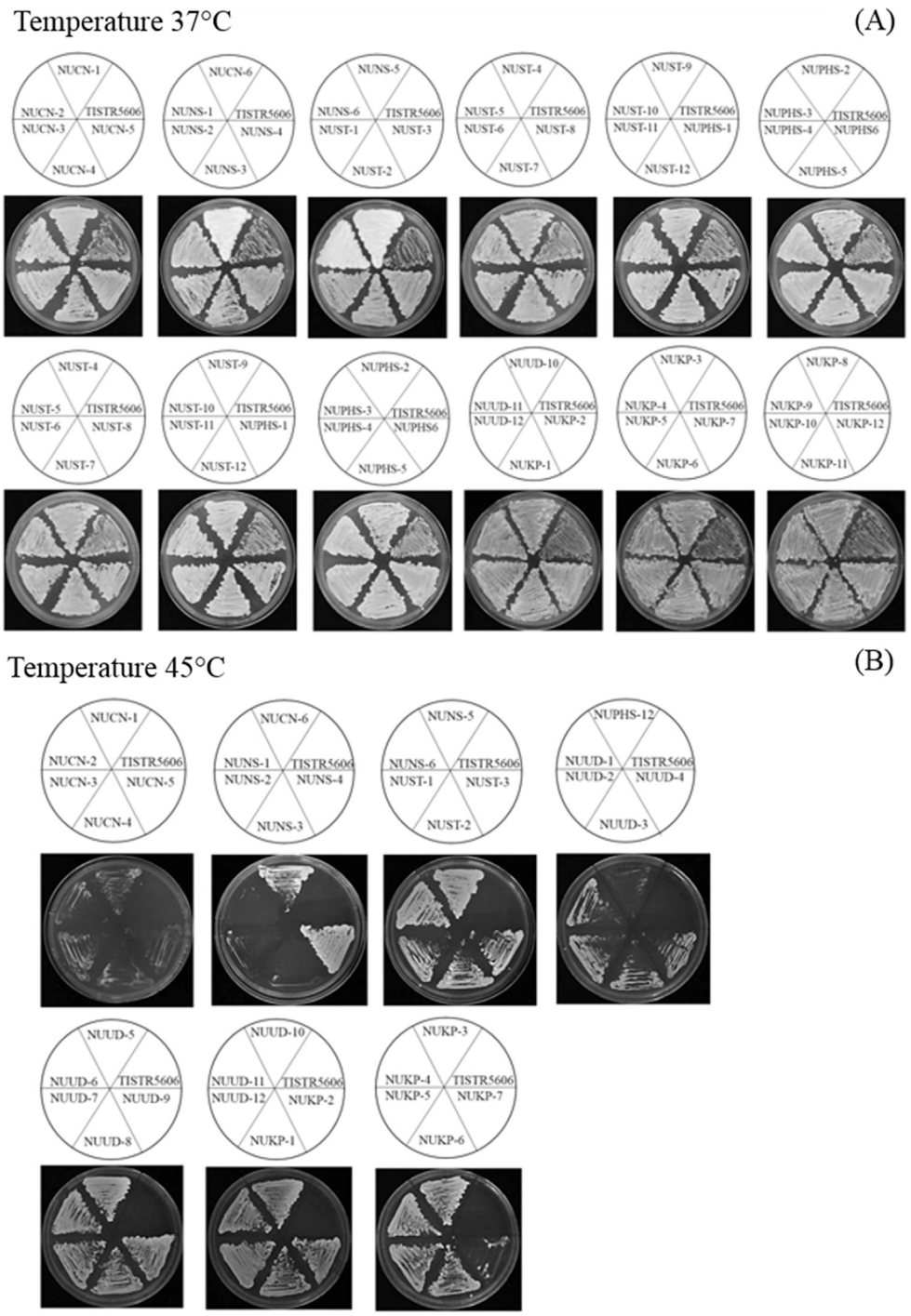

Fig. 1. Growth performance of 60 yeast isolates and *S. cerevisiae* TISTR 5606 after 72 hr incubation on yeast peptone dextrose agar plates under heat stress condition at: (A) 37 °C; (B) 45 °C.

Discussion

The most important key for success in the high temperature ethanol fermentation process is the utilization of potent thermotolerant yeasts, which could potentially overcome many obstacles (Auesukaree et al., 2012). Hence, thermophilic or thermotolerant microorganisms or both are of great interest for industrial applications. This study attempted to find newly identified thermotolerant yeast isolates from natural sources in Thailand. Heat and ethanol exposure are lethal stresses in yeast cells. Synthesizing heat-shock proteins after yeast cells encounter temperatures above 35 °C or ethanol levels higher than 4–6% (v/v) are responsive characteristics for strong induction (Piper, 1995). Several thermotolerant yeasts have been isolated using selection pressure by temperature and by ethanol concentration (Yuan et al., 2012). It has been reported that the effect of high temperatures and of ethanol concentrations greater than 3% directly affect microbial growth (Limtong et al., 2007). Therefore, an ethanol concentration of 4% (v/v) and incubation at 35 °C served as selection pressures for the isolation of thermotolerant yeasts in this study. The results

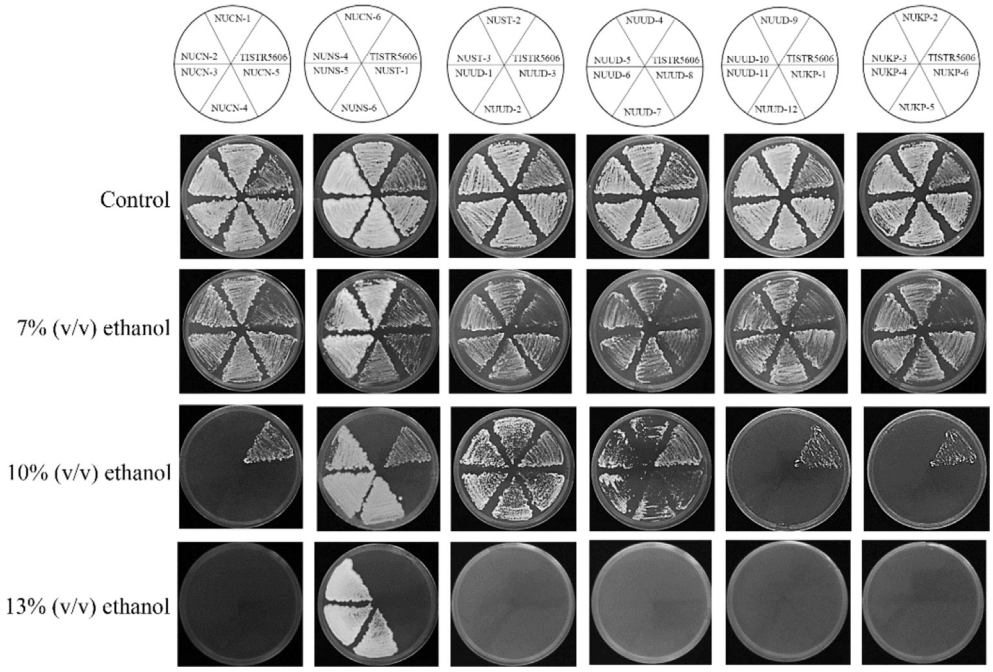

Fig. 2. Effect of ethanol stress on the cell growth of selected thermotolerant yeasts and *S. cerevisiae* TISTR 5606 on yeast peptone dextrose agar plates after incubation at 37 °C for 5 d.

Table 2
Ethanol production (mean ± SD) of thermotolerant yeast isolates at different incubation times and temperatures compared to the reference isolate *S. cerevisiae* TISTR 5606.

Temperature (°C)	Isolate	Ethanol concentration (g/L) after fermentation for		
		24 hr	36 hr	48 hr
40	NUNS–4	$58.78 \pm 0.51^{A*}$	$82.36 \pm 1.79^{A*}$	$88.60 \pm 0.75^{A*}$
	NUNS–5	$49.39 \pm 1.04^{B*}$	$76.02 \pm 0.33^{B*}$	$78.52 \pm 0.21^{B*}$
	NUNS–6	$53.16 \pm 0.59^{C*}$	$71.06 \pm 0.84^{C*}$	$77.97 \pm 0.65^{B*}$
	TISTR 5606	$34.37 \pm 0.74^{D*}$	$56.77 \pm 1.32^{D*}$	$65.37 \pm 0.82^{C*}$
45	NUNS–4	$37.78 \pm 1.39^{a*}$	$51.05 \pm 1.20^{a*}$	$54.30 \pm 0.97^{a*}$
	NUNS–5	$16.17 \pm 0.86^{b*}$	$37.73 \pm 1.46^{b*}$	$28.62 \pm 1.66^{b*}$
	NUNS–6	$31.03 \pm 1.32^{c*}$	$42.28 \pm 1.23^{c*}$	$44.04 \pm 0.92^{c*}$
	TISTR 5606	$3.27 \pm 0.65^{d*}$	$4.07 \pm 0.38^{d*}$	$3.96 \pm 0.47^{d*}$

* statistical difference between values of NUNS–4, NUNS–5 and NUNS–6 isolates, and *S. cerevisiae* TISTR 5606 for the same conditions of temperature and incubation time. ^{abcd} or ^{ABCD} values bearing different superscripts within the same column of each temperature are significantly ($p < 0.05$) different based on Duncan's multiple range test.

obtained in terms of screening thermotolerance were consistent with previous studies which have reported that the thermotolerant yeasts, including S1–2, S6–1, S10–2, KKU–TH33, KKU–TH43, KKU–VN8, KKU–VN20 and KKU–VN27, also could grow in temperatures ranging up to 45 °C (Buddiwong et al., 2014; Techaparin et al., 2017). However, the growth ability of yeasts under these high temperatures is dependent on differential yeast strains and individual specific growth rates.

During the fermentation process, yeast cells must encounter various stresses such as heat and ethanol concentration (Kitichantaropas et al., 2016). Cellular mechanisms are involved in protecting the yeast cell from various physical and chemical stressors. Ethanol concentrations higher than 10% (v/v) can negatively affect yeast cell metabolism and growth and can damage the cell wall, decreasing the specific growth rate, increasing cell death or changing the permeability of the plasma membrane and the transport systems (Stanley et al., 2010; Costa et al., 2014; Techaparin et al., 2017). Therefore, yeast cells that are able to tolerate ethanol concentrations above 10% (v/v) are necessary for use in the ethanol industry. The differences in ethanol or stress tolerance might be due to the differences in natural sources for the isolation and type of yeast strains. Previously, Chamnipa et al. (2017) reported that the thermotolerant yeast *P. kudriavzevii* RZ8–1 could grow in a yeast extract and malt extract (YM) agar containing 12% (v/v) ethanol at 35 °C. Abe et al. (2009) reported *S. cerevisiae* wild type W303–1A grew slightly on yeast extract peptone adenine sulfate dextrose (YPAD) agar containing 1% yeast extract, 2% peptone, 0.02% adenine sulfate and 2% glucose which contained 8% (v/v) ethanol but could not grow on agar containing 10% or 13% (v/v) ethanol at 30 °C. Those authors found that expression of a proofreading-deficient DNA polymerase δ, *pol3–01* gene, in *S. cerevisiae* W303–1A tolerated in YPAD agar containing 13% ethanol which was higher than the wild-type strain, *S. cerevisiae* W303–1A. Buddiwong et al. (2014) found 15 thermotolerant yeast cells were able to tolerate a YM agar containing up to 10% (v/v) ethanol concentration and incubation at 30 °C. The current results suggested that three thermotolerant yeasts (NUNS–4, NUNS–5 and NUNS–60 were not only able to tolerate heat stress (45 °C) but also a high ethanol concentration (13% v/v). This finding was in accordance with that of Costa et al. (2014) who explained the "cross–tolerance" hypothesis in which one type of stress enhances the protection against other stresses. Further investigations are

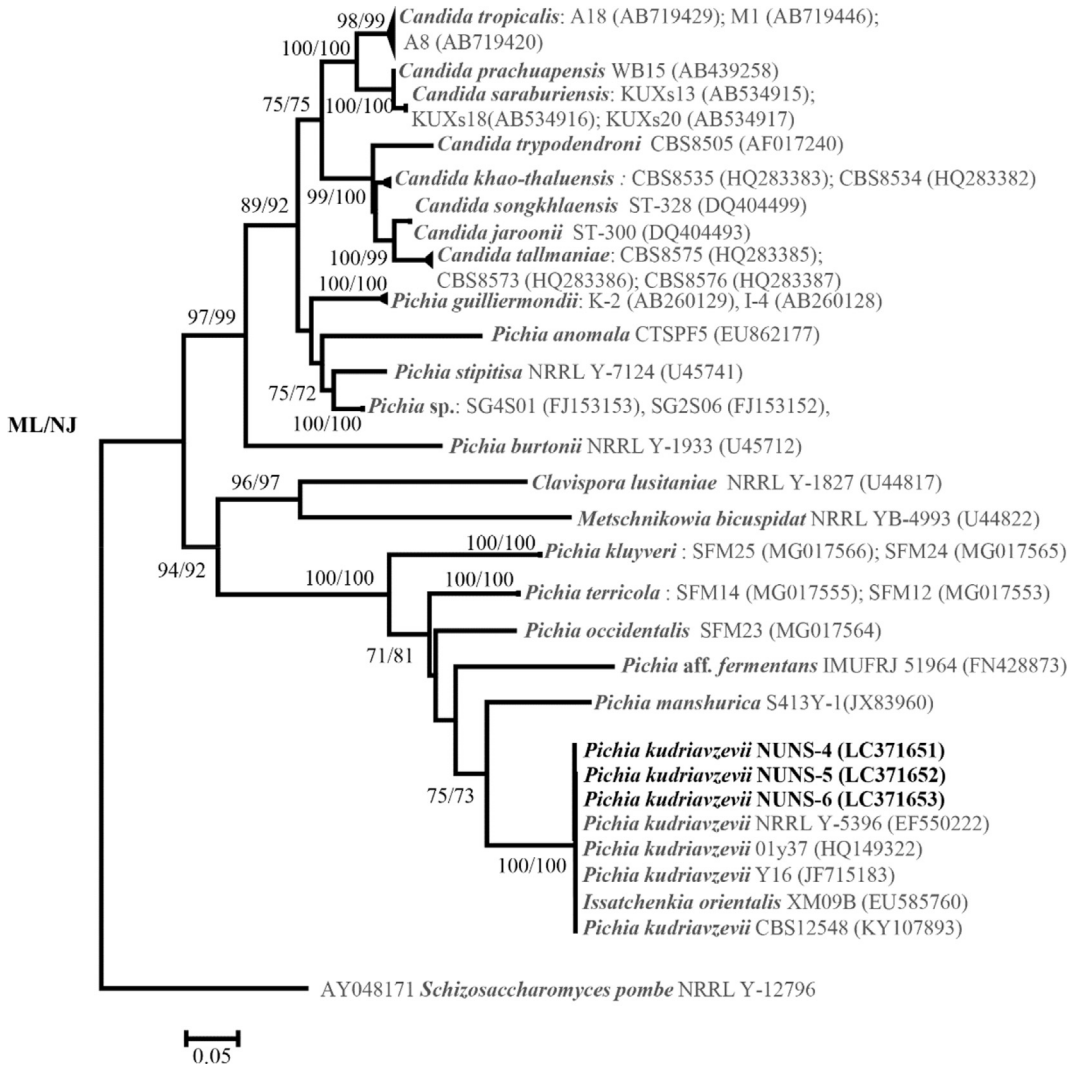

Fig. 3. Maximum likelihood tree based on the D1/D2 domain of the large subunit (26S) rDNA, where bootstrap percentages (>50%) of maximum likelihood (ML) and neighbor-joining (NJ) analyses are indicated, respectively, from left to right for each branch and *Schizosaccharomyces pombe* NRRL Y−12796 was used as the outgroup species, while Thai *Pichia kudriavzevii* strains determined in this study are marked with bold letters.

necessary to support the cross-tolerance hypothesis for the isolates of NUNS.

There have been several thermotolerant yeasts which have produced ethanol such as *S. cerevisiae*, *K. marxianus*, *Pichia* sp. and *Candida* sp. (Limtong et al., 2007; Auesukaree et al., 2012; Buddiwong et al., 2014; Costa et al., 2014). The newly isolated NUNS−4 is an outstandingly competent yeast for high-temperature ethanol fermentation in the ethanol production industry. During the fermentation process at high temperature, several factors such as temperature and the glucose concentration have effects on the growth of yeast cells and the ethanol concentration. Costa et al. (2014) reported that the *K. marxianus* UFV−3 strain grew better at a high glucose concentration than at a low glucose concentration at 45 °C. *S. cerevisiae* LBM−1, PE−2 and CAT−1 did not grow at 42 °C in 2% (w/v) glucose; growth only occurred in glucose concentrations above 2% (w/v) in the yeast extract peptone broth. Therefore, it is possible that the high glucose concentration protected the cell from stress caused by increased temperature. Moreover, previous study also found that above 40 °C, yeast viability decreases considerably, which affects ethanol production, so that together, glucose concentration and temperature factors affect ethanol production (Costa et al., 2014).

Based on the D1/D2 domain of 26S rDNA sequences, the current results were identical to the topologies of Chamnipa et al. (2017) and Techaparin et al. (2017). The results revealed that three isolates in the current study can be grouped in the *P. kudriavzevii* clade. This species was previously known as *Issatchenkia orientalis* and it has often been characterized and stated that *P. kudriavzevii* acts as a robust and multistress-tolerant yeast resistant to such stresses as acidic conditions and high temperature or salt content (Koutinas et al., 2016; Chamnipa et al., 2017). Some reports have indicated that the characterization of *P. kudriavzevii* makes it applicable for industrial processes (Dhaliwal et al., 2011; Yuangsaard et al., 2013; Koutinas et al., 2016; Chamnipa et al., 2017; Joshi and Patel, 2017; Techaparin et al., 2017). The current report has also provided important data regarding novel *P. kudriavzevii* NUNS−4, NUNS−5 and NUNS−6 strains for use in industrial applications. A comparative analysis of the ethanol production by the newly isolated *P. kudriavzevii* NUNS and other yeast isolates as reported in the previous literature is demonstrated in Table 3.

In the current study, 30 thermotolerant yeasts from soil in sugarcane fields were successfully isolated. Based on results of the current study, it has been shown that *P. kudriavzevii* NUNS strains were not only tolerant to heat and ethanol stresses but also were

Table 3
Comparison of ethanol tolerance and ethanol production by *P. kudriavzevii* NUNS–4, NUNS–5 and NUNS–6 and other yeast isolates.

Microorganism	Source of sample	Level of ethanol tolerance (volume per volume %)	Substrate concentration	Maximal ethanol concentration (g/L)	Temperature (°C)	Reference
I. orientalis S6–1	Soil	10	16% (w/v) glucose	53.56	40	Buddiwong et al. (2014)
S. cerevisiae KKU–VN8	Fruit, flower, alcoholic beverage and soil	10	20% (w/v) glucose	89.32	40	Techaparin et al. (2017)
S. cerevisiae C3867	Fruit	No data	10% (w/v) glucose	38.80	41	Auesukaree et al. (2012)
K. marxianus BUNL–21	Fruits, vegetables, leaves and soils of Lao People's Democratic Republic	8	20% (w/v) xylose	2.91	30	Nitiyon et al. (2016)
P. kudriavzevii EM12	Muskmelon	12	10% (w/v) sucrose	38.00	40	Joshi and Patel (2017)
P. kudriavzevii S10–2	Soil	10	16% (w/v) glucose	57.99	40	Buddiwong et al. (2014)
P. kudriavzevii DMKU 3–ET15	Fermented pork sausage	No data	16% (w/v) glucose	78.60	40	Yuangsaard et al. (2013)
P. kudriavzevii RZ8–1	Plant orchard	12	16% (w/v) glucose	69.85	40	Chamnipa et al. (2017)
P. kudriavzevii KVMP10	Orange peel	No data	10% (w/v) orange peel hydrolysate	54.00	42	Koutinas et al. (2016)
P. kudriavzevii NUNS–4	Soil	13	16% (w/v) glucose	88.60	40	This study
P. kudriavzevii NUNS–5	Soil	13	16% (w/v) glucose	78.52	40	This study
P. kudriavzevii NUNS–6	Soil	13	16% (w/v) glucose	77.97	40	This study

able to produce a high ethanol concentration at high temperature. The ethanol production capabilities of these three *P. kudriavzevii* isolates (NUNS–4, NUNS–5 and NUNS–6) had high fermentation performance at high temperature. The current results have presented important information with regard to these candidate yeasts for utilization as ethanol producers, which would help to improve the productive efficiency of the biotechnological industry in the near future. To gain further insight to improve ethanol yields from microorganisms, several factors require further determination in the molecular mechanisms of genes involved in metabolic processes and oxidative stress defense as well as factors that affect ethanol yield, such as the pH level and the type of substrate and carbon source.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This research was financially supported by a grant from Research Project No. R2561B065, Naresuan University, Thailand. The authors are grateful to Mr. Clifford Sloane for editing the English in an earlier version of the manuscript.

References

Abe, H., Fujita, Y., Takaoka, Y., Kurita, E., Yano, S., Tanaka, N., Nakayama, K.–i., 2009. Ethanol-tolerant *Saccharomyces cerevisiae* strains isolated under selective conditions by over–expression of a proofreading–deficient DNA polymerase δ. J. Biosci. Bioeng. 108, 199–204.

Apiwatanapiwat, W., Rugthaworn, P., Vaithanomsat, P., et al., 2013. Ethanol production at high temperature from cassava pulp by a newly isolated *Kluyveromyces marxianus* strain, TISTR 5925. AIMS Energy 1, 1–14.

Auesukaree, C., Koedrith, P., Saenpayavai, P., et al., 2012. Characterization and gene expression profiles of thermotolerant *Saccharomyces cerevisiae* isolates from Thai fruits. J. Biosci. Bioeng. 114, 144–149.

Banat, I.M., Nigam, P., Singh, D., Marchant, R., McHale, A.P., 1998. Review: ethanol production at elevated temperatures and alcohol concentrations: Part I – yeasts in general. World J. Microbiol. Biotechnol. 14, 809–821.

Buddiwong, S., Thanonkeo, S., Phetsom, J., Jaisil, P., Thanonkeo, P., 2014. Screening of thermotolerant yeast isolated from sugarcane plantations in northeastern part of Thailand. KKU Res. J. 19, 217–223.

Chamnipa, N., Thanonkeo, S., Klanrit, P., Thanonkeo, P., 2017. The potential of the newly isolated thermotolerant yeast *Pichia kudriavzevii* RZ8–1 for high-temperature ethanol production. Braz. J. Microbiol. 49, 378–391.

Costa, D.A., De Souza, C.J.A., Costa, P.S., et al., 2014. Physiological characterization of thermotolerant yeast for cellulosic ethanol production. Appl. Microbiol. Biotechnol. 98, 3829–3840.

Dhaliwal, S.S., Oberoi, H.S., Sandhu, S.K., Nanda, D., Kumar, D., Uppal, S.K., 2011. Enhanced ethanol production from sugarcane juice by galactose adaptation of a newly isolated thermotolerant strain of *Pichia kudriavzevii*. Bioresour. Technol. 102, 5968–5975.

Edgardo, A., Carolina, P., Manuel, R., Juanita, F., Baeza, J., 2008. Selection of thermotolerant yeast strains *Saccharomyces cerevisiae* for bioethanol production. Enzym. Microb. Technol. 43, 120–123.

Guindon, S., Dufayard, J.–F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. 59, 307–321.

Hesham, A.–L., Mohamed, H., 2011. Molecular genetic identification of yeast strains isolated from Egyptian soils for solubilization of inorganic phosphates and growth promotion of corn plants. J. Microbiol. Biotechnol. 21, 55–61.

Joshi, B.H., Patel, D.K., 2017. Screening and characterization of newly isolated thermotolerant and ethanogenic strain of *Pichia kudriavzevii*. Int. J. Agric. Environ. Biotechnol. 10, 115–123.

Kaewkrajay, C., Dethoup, T., Limtong, S., 2014. Ethanol production from cassava using a newly isolated thermotolerant yeast strain. Sci. Asia 40, 268–277.

Kitichantaropas, Y., Boonchird, C., Sugiyama, M., Kaneko, Y., Harashima, S., Auesukaree, C., 2016. Cellular mechanisms contributing to multiple stress tolerance in *Saccharomyces cerevisiae* strains with potential use in high-temperature ethanol fermentation. AMB Express 6, 1–14.

Koutinas, M., Patsalou, M., Stavrinou, S., Vyrides, I., 2016. High temperature alcoholic fermentation of orange peel by the newly isolated thermotolerant *Pichia kudriavzevii* KVMP10. Lett. Appl. Microbiol. 62, 75–83.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.

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.

Limtong, S., Yongmanitchai, W., Kawasaki, H., Fujiyama, K., 2009. *Wickerhamomyces edaphicus* sp. nov. and *Pichia jaroonii* sp. nov., two ascomycetous yeast species isolated from forest soil in Thailand. FEMS Yeast Res. 9, 504–510.

Lõoke, M., Kristjuhan, K., Kristjuhan, A., 2011. Extraction of genomic DNA from yeasts for PCR–based applications. Biotechniques 50, 325–328.

Nitiyon, S., Keo–Oudone, C., Murata, M., Lertwattanasakul, N., Limtong, S., Kosaka, T., Yamada, M., 2016. Efficient conversion of xylose to ethanol by stress-tolerant *Kluyveromyces marxianus* BUNL–21. SpringerPlus 5, 1881–1886.

Okonechnikov, K., Golosova, O., Fursov, M., et al., 2012. Unipro UGENE: a unified bioinformatics toolkit. Bioinformatics 28, 1166–1167.

Piper, P.W., 1995. The heat shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. FEMS Microbiol. Lett. 134, 121–127.

Pongcharoen, P., Kawano-Kawada, M., 2018. Identification and characterization of *Candida tropicalis* isolated from soil of sugarcane plantation in Thailand for ethanol production. Asia Pac. J. Sci. Technol. 23, 1–9.

Siedlarz, P., Sroka, M., Dyląg, M., Nawrot, U., Gonchar, M., Kus–Liśkiewicz, M., 2016. Preliminary physiological characteristics of thermotolerant *Saccharomyces cerevisiae* clinical isolates identified by molecular biology techniques. Lett. Appl. Microbiol. 62, 277–282.

Sree, N.K., Sridhar, M., Suresh, K., Banat, I.M., Rao, L.V., 2000. Isolation of thermotolerant, osmotolerant, flocculating *Saccharomyces cerevisiae* for ethanol production. Bioresour. Technol. 72, 43–46.

Stanley, D., Bandara, A., Fraser, S., Chambers, P.J., Stanley, G.A., 2010. The ethanol stress response and ethanol tolerance of *Saccharomyces cerevisiae*. J. Appl. Microbiol. 109, 13–24.

Talukder, A.A., Easmin, F., Mahmud, S.A., Yamada, M., 2016. Thermotolerant yeasts capable of producing bioethanol: isolation from natural fermented sources, identification and characterization. Biotechnol. Biotechnol. Equip. 30, 1106–1114.

Techaparin, A., Thanonkeo, P., Klanrit, P., 2017. High-temperature ethanol production using thermotolerant yeast newly isolated from Greater Mekong subregion. Braz. J. Microbiol. 48, 461–475.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position–specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Wu, W.H., Hung, W.C., Lo, K.Y., Chen, Y.H., Wan, H.P., Cheng, K.C., 2016. Bioethanol production from taro waste using thermo-tolerant yeast *Kluyveromyces marxianus* K21. Bioresour. Technol. 201, 27–32.

Yuan, W.J., Chang, B.L., Ren, J.G., Liu, J.P., Bai, F.W., Li, Y.Y., 2012. Consolidated bioprocessing strategy for ethanol production from Jerusalem artichoke tubers by *Kluyveromyces marxianus* under high gravity conditions. J. Appl. Microbiol. 112, 38–44.

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.