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Identification and characterization of *Candida tropicalis* isolated from soil of sugarcane field in Thailand for ethanol production

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Abstract

Thermotolerant and ethanol-producing yeasts are mainly used in numerous industrial applications, such as production of bioethanol. In this study, 12 novel thermotolerant yeast strains were isolated using enrichment technique with 4% (v/v) ethanol. The growth phenotype and ethanol fermentation activity under stress conditions were compared with that of Thai industrial strain *Saccharomyces cerevisiae* TISTR5339. All isolated strains showed ability to grow at 42°C. Among them, five isolates were found to be tolerant up to 20% (v/v) ethanol and six isolates demonstrated higher tolerance both to high temperature (40°C) and ethanol concentration up to 10% (v/v). The specific growth rates (μ) at high temperature of all strains ranged between 0.470-0.577 (h^{-1}) at 40°C, which were greater than that of the reference strain. Isolated strain KPC6, showed higher productivity compared to the reference strain by converting glucose to ethanol at 14.422 g/L in 12 h and 45.232 g/L in 24 h at 42°C. Phylogenetic analysis based on the sequences of D1/D2 domain of 26S rDNA revealed that all strains were *Candida tropicalis*. Results from this study have shown the potential of Thai *C. tropicalis* KPC strains in industrial ethanol production.

Keywords: Candida tropicalis, thermotolerant, ethanol tolerant, ethanol production

1. Introduction

In the industrial process to convert sugar-based biomass to bioethanol, heat is released due to exothermic reactions which increases the temperature of fermentation to approximately 50°C, whereas most fermentative microorganisms have an optimum temperature for growth between 30°C to 37°C [1-3]. The desirable traits required for efficient production of bioethanol such as tolerance to high temperature and ethanol concentration, are major properties of microorganisms which are exploited in industrial processes. These tolerance features are likely to be useful not only for reduction in cooling costs but also for improvement of reactions and avoidance of microbial contamination.

Among the microorganisms which are used for industrial purposes, yeasts are the most commonly used ones for biomass, bioethanol production and beverage industry, since they exhibit distinctive characteristics when grown under stress conditions [4-6]. To achieve fermentation at high temperature, thermotolerant and ethanol-producing yeasts have come to be recognized as good candidates. Currently, extensive screening and identification of microorganisms from distinct ecological niches such as tropical and subtropical regions have been carried out [7,8]. *Kluyveromyces marxianus* BUNL-21 isolated in Laos was indicated to be tolerant to various stresses including high temperature, ethanol, and hydrogen peroxide [9]. Newly isolated *Pichia kudriavzevii* DMKU 3-ET1 from soil and traditional fermented foods in Thailand could be employed for ethanol production at high

temperatures up to 45°C [10]. Thermotolerant isolates obtained from Thai fruits, *Saccharomyces cerevisiae (*C3723, C3751, and C3867*)*, were able to grow and produce ethanol (38 g/L) at 41°C [7]. Considering previous studies, Thailand, one of the tropical countries with high ambient temperature throughout the year, is a place of interest for isolation of thermotolerant microbial resources.

Reports have shown that some *Candida tropicalis* are capable of growth and fermentation at higher temperature, and useful for the commercial ethanol production. *C. tropicalis* strain No. 10 isolated from chemically contaminated soil was capable of phenol degradation between 20°-42°C [11]. *C. tropicalis* YMEC14 is a potential microorganism for the production of ethanol as it is capable of fermenting starch at a low rate [12]. However, no report has described in detail *C. tropicalis* growth, physiology and response to heat and ethanol stresses.

The objective of this study was identification and characterization of useful yeasts isolated from soil of sugarcane field in Thailand. In this study, we estimate the growth property of isolated yeast strains under conditions with high temperature and high ethanol concentration, and capability of ethanol production of these strains under high temperature was evaluated.

2. Materials and Methods

2.1 Isolation of yeasts

Soil samples from sugarcane plantations were randomly collected and subjected to isolation of yeasts. Isolation was carried out by enrichment technique using YPD (1% yeast extract, 2% peptone, and 2% glucose) supplemented with 4% (v/v) ethanol, 0.025% sodium propionate (P1880, Sigma), and 0.02% chloramphenicol (C0378, Sigma). The living cells were streaked on YPD agar plate and incubated at 37°C until colonies appeared. Isolated strains were designated as KPC strains. For long-term storage, the purified yeast strains were suspended in YPD medium supplemented with 25% glycerol and maintained at -80°C. The reference strain, *S. cerevisiae* strain TISTR5339, an industrial ethanol-producing strain, was purchased from Thailand Institute of Scientific and Technological Research, Bangkok, Thailand.

2.2 Examination of the growth sensitivity of cells to thermo- or ethanol- stress.

All strains used in this study were cultured in YPD medium at 30°C until they reached an $O.D._{660}$ of 1.0, and cells were diluted to 10^{-1} steps and subjected to 10-fold serially-diluted medium. Three µL of the appropriated dilution culture was spotted onto YPD agar plate and incubated at different temperatures (30°, 37°, 40°, 42°, and 45°C). To determine the growth ability with different ethanol-supplemented levels at high temperature, ethanol was added to YPD agar at 10, 15, and 20% (v/v). The plates were incubated for three days, and the growth of the cells was monitored. Each experiment was repeated two times.

2.3 Estimation of the specific growth rate

Yeast isolates were pre-cultivated in YPD medium at 30°C, and were inoculated into fresh YPD medium to give an $O.D._{660}$ of 0.05 for further cultivation with shaking at 30°, 37°, 40°, 42°, and 45°C. Optical densities at 660 nm were recorded at 1 h intervals up to 24 h. Specific growth rates were calculated by linear regression of the values obtained by the Napierian logarithm of the $O.D._{660}$ during the exponential growth phase. The specific growth rate is the slope of the line which was obtained from five points of a plot of $O.D._{660}$ versus time (h) [1].

2.4 Analysis of ethanol fermentation ability at high temperatures

Yeast cells were aerobically pre-cultivated to exponential phase in YPD medium at 30°C, and then were inoculated to 30 mL fresh medium of YPD containing 16% (w/v) glucose as a carbon source at the initial $O.D._{660}$ value of 0.1 before further cultivation at specific temperature of 40°C and 42°C. A portion of fermentation broth was withdrawn from each flask after 12 h and 24 h incubation; then, centrifuged at 16,200× *g* for 10 min at 4°C. The supernatant were collected and subjected to measure the concentration of ethanol by a gas chromatography GC-14B apparatus (Shimadzu, Kyoto, Japan) [13]. The variance analysis (ANOVA) was used to analyze data to determine the significant differences between the isolated and the reference strains.

2.5 Identification of the yeast strain

Genomic DNA was extracted from the yeast cells and subjected to PCR in order to amplify the D1/D2 domain of the larger subunit (LSU) 26S rDNA region using the primer NL-1 (5‘-GCATATCAATAAGCGGAGGAAAAG-3‘) and NL-4 (5‘-GGTCCGTGTTTCAAGACGG-3‘) [10,14]. The

PCR products were sequenced by using ABI BigDye Terminator ver. 3.1 Cycle sequencing kit and ABI 3130 DNA Analyzer (Applied Biosystems, California, USA). The obtained nucleotide sequences were analyzed using the software UGENE [15]. The D1/D2 LSU rDNA sequences from this work and those of other thermotolerant yeasts retrieved from the database were aligned using the CLUSTAL W software [16]. The phylogenetic trees were constructed using the Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods, and topology of the trees was tested by performing 1,000 replicates bootstrap replications. The NJ tree was generated from evolutionary distance data calculated from the p distance method implemented in MEGA7 [17]. The ML tree was performed with the GTR+G+I model using PhyML 3.0 [18]. In this study, the D1/D2 LSU sequences of the KPC1 to KPC12 strains were deposited in GeneBank as LC218100 to LC218111.

3. Results and Discussion

Among the various procedures for isolating yeasts capable of producing ethanol at high temperature, isolation from natural habitats has been one of the most effective ways [19]. Recently, several thermotolerant yeast strains were obtained by an enrichment isolation technique at a high temperature of 45°-50°C. Kiran *et al*. (2000) reported the isolation of *S. cerevisiae* strains which are capable of ethanol fermentation at elevated temperatures and are also tolerant to high concentrations of sugar [20]. Limtong *et al*. (2007) was successful in isolating *K. marxianus* DMKU 3-1042, from soil and water samples of sugarcane plantations by an enrichment technique containing 4% (v/v) ethanol [21]. In order to obtain yeast strains that are tolerant to high concentrations of ethanol and high temperatures, a cultured medium should be supplemented with ethanol concentrations of at least 3% and incubated at 35°C [8,10,21]. In this study, an enrichment isolation technique with 4% (v/v) ethanol at 37°C was performed. A total of 12 yeast strains from sugarcane plantation soil were isolated. The isolated strains were designated KPC1 to KPC12. To evaluate heat tolerance of isolated strains, the growth phenotypes of strains were estimated by spot the cells on YPD agar under control (30°C) or heat stress 37°, 40°, 42°, or 45°C (Figure 1). The growth at 30°C and 37°C was very similar for all strains when compared with the Thai industrial strain, TISTR5339 after three days incubation. All KPC strains tested here could grow at 40°C whereas TISTR5339 could not. At the higher temperature of 42°C, some strains (KPC1, KPC3, KPC4, KPC5, and KPC6) showed significant tolerance to heat stress compared with the other isolates and TISTR5339. However, all of the isolated strains hardly grew at 45°C (data not shown).

The effect of ethanol on growth was also evaluated (Figure 1). All strains tested in this study could grow in media containing 10% and 15% (v/v) ethanol at 30°C and 37°C. Among 12 isolates, five strains (KPC1, KPC2, KPC3, KPC4, and KPC8) grew on agar plates containing ethanol up to 20% (v/v) at 30°C, as same extent as the reference strain TISTR5339. Six strains (KPC1, KPC2, KPC3, KPC4, KPC5, and KPC6) exhibited their ability to grow at 40°C supplemented with 10% (v/v) ethanol. The observed resistance to ethanol was almost completely lost at 42°C. The tolerance to ethanol was decreased with increasing temperature. It is possible that membrane damage and protein denaturation and aggregation can be caused by high temperature. High ethanol concentration can result in the disorganization of the structure of cell wall, malfunctioning of transport systems, such as glucose transport system [22], cause a change in the permeability of the plasma membrane and perturb protein conformation [1,23]. Interestingly, the isolates which could grow at higher temperature, such as KPC1, KPC3, KPC4, KPC5, and KPC6, also showed higher tolerance to ethanol than other isolates (Figure 1, 40°C with 10% ethanol and 42°C). The phenotype of these thermo- and ethanol- tolerant strains may be explained by "cross-tolerance", in which one type of stress assists partial protection against other stresses [22,24]. Because the induction of cross-tolerance to some stresses should be related to multiple factors such as heat-shock proteins, cross-talk between signal transduction processes, or growth-regulated genes [25], further investigations are necessary to support the cross-tolerance hypothesis for the KPC isolates.

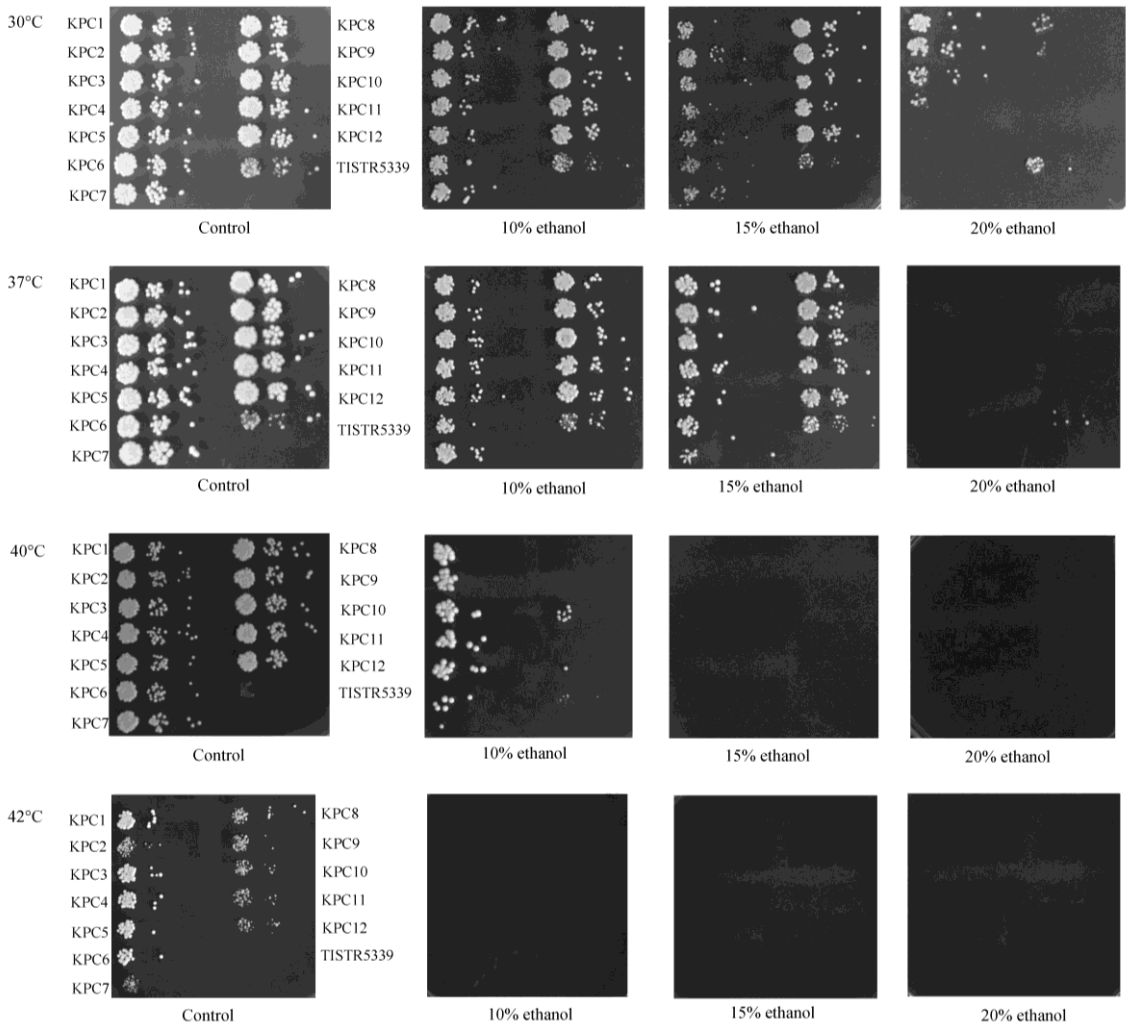

Figure 1 Growth ability of Thai KPC isolated strains and *S. cerevisiae* TISTR5339 under thermo- and ethanol-stress conditions. The reference (TISTR5339) or isolated KPC strains were cultured in YPD medium and spotted onto YPD plates with or without ethanol at various concentrations as described in the "Materials and Methods" section. The plates were incubated at indicated temperature for three days.

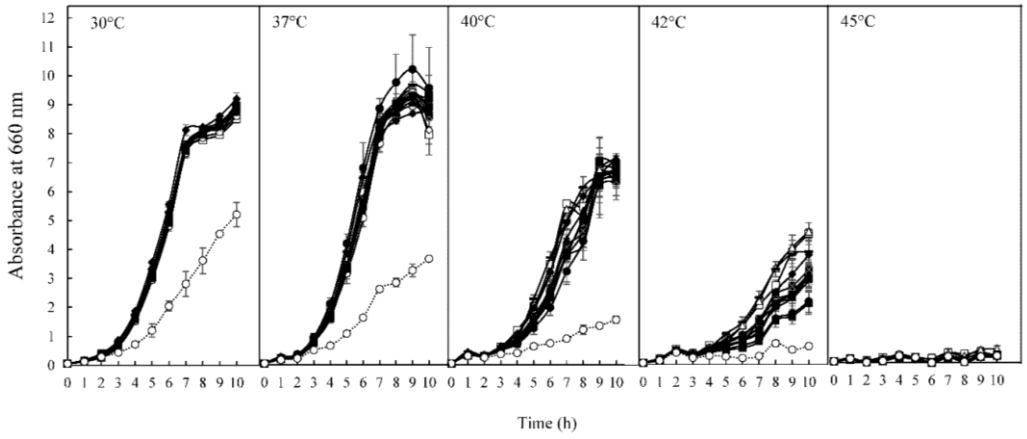

Figure 2 Growth properties of the Thai KPC strains and *S. cerevisiae* TISTR5339 in liquid media under different temperatures. The cells were pre-cultivated at 30°C to early logarithmic phase and were inoculated in fresh YPD media at $O.D._{660}$ of 0.05, and started to culture at indicated temperature. The growth of cells was monitored by measuring $O.D._{660}$. □; KPC1, ○; KPC2, ◇; KPC3, Δ; KPC4, ×; KPC5, ✳; KPC6, -; KPC7, ■; KPC8, ●; KPC9, ◆; KPC10, ▲; KPC11, ✚; KPC12, and --○--; TISTR5339. Error bars represent the mean ± standard deviation from three independent experiments.

The yeast strain which can grow at 40°C with a high specific growth rate ($\mu \geq 0.30\ h^{-1}$) have been classified as thermotolerant yeasts [20,26]. We determined the specific growth rate (μ) to evaluate thermotolerance of the isolated strains. The specific growth rates of all KPC isolates at 30°C were 0.557-0.608 (h^{-1}) (Table 1). With increasing temperature to 37°C, the growth of KPC strains were not impaired (Figure 2) with the specific growth rates ranged from 0.542 to 0.593 (h^{-1}). At 40°C, the growth rates of all KPC strains were ranging between 0.470-0.577 (h^{-1}), which were greater than that of the reference strain. The growth rate of all tested strains slightly decreased at 42°C than at 30°C and 37°C. It was noticed that all strains isolated in this study showed higher μ values compared to TISTR5339 at 40°C (Table 1), suggesting that the KPC strains are thermotolerant ones. Moreover, the KPC strains seem to be more tolerant than the other thermotolerant strains reported previously, such as *S. cerevisiae* isolates (C3723, C3751, and C3867)[7] and *K. marxianus* UFV-3 [1]. The KPC isolated yeasts can be useful for ethanol fermentation at high temperature.

Table 1 Specific growth rate (μ) values of the isolated KPC strains compared to that of the reference strain TISTR5339. μ value presented was averaged value of triplication based on three independent experiments. Data were shown as mean ± standard deviation. One way ANOVA was used to analyze the significant difference between the isolated stains and the reference one. The μ value of all isolated strain within the same column are significantly different at the level of 0.01.

Yeast strain	Specific growth rate (μ, h^{-1})			
	Temperature (°C)			
	30	37	40	42
KPC1	0.600 ± 0.006	0.556 ± 0.000	0.544 ± 0.005	0.423 ± 0.001
KPC2	0.592 ± 0.007	0.555 ± 0.007	0.517 ± 0.007	0.334 ± 0.007
KPC3	0.585 ± 0.006	0.567 ± 0.014	0.511 ± 0.024	0.384 ± 0.030
KPC4	0.582 ± 0.003	0.560 ± 0.005	0.577 ± 0.019	0.448 ± 0.011
KPC5	0.590 ± 0.021	0.583 ± 0.035	0.526 ± 0.019	0.385 ± 0.011
KPC6	0.608 ± 0.011	0.591 ± 0.021	0.504 ± 0.003	0.353 ± 0.006
KPC7	0.560 ± 0.001	0.542 + 0.012	0.552 ± 0.007	0.382 ± 0.037
KPC8	0.585 ± 0.005	0.584 ± 0.028	0.510 ± 0.007	0.364 ± 0.010
KPC9	0.583 ± 0.012	0.560 ± 0.012	0.470 ± 0.009	0.325 ± 0.019
KPC10	0.557 ± 0.005	0.573 ± 0.030	0.552 ± 0.012	0.370 ± 0.002
KPC11	0.565 ± 0.012	0.580 ± 0.013	0.522 ± 0.024	0.347 ± 0.010
KPC12	0.589 ± 0.002	0.593 ± 0.010	0.513 ± 0.018	0.338 ± 0.017
TISTR5339	0.325 ± 0.032	0.471 ± 0.007	0.267 ± 0.003	0.183 ± 0.020

Table 2 Ethanol production by the KPC strains and TISTR5339. Values presented was averaged value of triplication based on three independent experiments. Data were expressed as mean ± standard deviation. One way ANOVA was used to determine the statistically significant difference; * indicates statistical difference between values of KPC strains and TISTR5339 for the same condition; significance level is 0.05

Temperature (°C)	Strains	Ethanol concentration (g/L)	
		12h	24h
40	KPC1	33.348 ± 1.298	50.651± 2.089
	KPC2	23.695 ± 2.289	40.455 ± 6.69
	KPC3	29.521 ± 3.094	51.151 ± 3.385
	KPC4	31.251 ± 5.993	50.999 ± 1.597
	KPC5	26.282 ± 5.384	63.972 ± 5.177
	KPC6	36.787 ±3.112	51.067 ± 0.581
	KPC7	29.58 ± 2.77	64.245 ± 0.168
	KPC8	35.979 ± 1.252	54.301 ± 7.181
	KPC9	31.40 ± 3.103	48.795 ± 0.798
	KPC10	31.626 ± 2.895	56.714 ± 4.586
	KPC11	34.26 ± 0.47	47.447 ± 6.120
	KPC12	39.117 ± 2.330	44.052 ± 2.913
	TISTR5339	34.455 ± 2.128	86.310 ± 3.380
42	KPC1	3.224 ± 0.20	28.739 ± 4.815
	KPC2	0.978 ± 0.255	28.385 ± 2.456
	KPC3	2.570 ± 0.608	34.668 ± 6.944
	KPC4	2.300 ± 1.881	23.375 ± 0.581
	KPC5	4.355 ±1.739	22.947 ± 4.798
	KPC6	14.422 ± 3.693*	45.232 ± 2.583*
	KPC7	1.687 ± 0.531	29.555 ± 5.789
	KPC8	5.232 ± 1.242	30.922 ± 5.833
	KPC9	3.133 ± 0.003	59.186 ± 2.577*
	KPC10	3.416 ± 0.764	48.885 ± 5.365*
	KPC11	3.379 ± 0.936	34.512 ± 4.635
	KPC12	2.333 ± 0.283	34.539 ± 3.756
	TISTR5339	3.373 ± 0.117	35.528 ± 1.015

Ethanol productivity of the KPC strains and *S. cerevisiae* TISTR5339 were investigated in fermentation medium with 16% (w/v) glucose at 40°C and 42°C (Table 2). After 12 h fermentation at 40°C, the KPC strains, except for KPC2 and KPC5, produced almost equal levels of ethanol compared with TISTR5339. When incubated at 42°C, the production of ethanol by the strain TISTR5339 decreased significantly. However, the isolated strains, KPC6, KPC9, and KPC10, retained the ethanol productivity at higher level than the reference stain. After 24 h incubation, significantly higher levels of ethanol production at 42°C were found with the strains KPC6 (45.232 ± 2.583 g/L), KPC9 (59.186 ± 2.577 g/L), and KPC10 (48.885 ± 5.365 g/L), while TISTR5339 produced much less ethanol (35.528 ± 1.015 g/L). From the results described above, newly thermotolerant yeast strain KPC6 can be a good candidate for ethanol production at high temperature.

Currently, several studies have distinguished the yeast species using molecular identification based on the D1/D2 domain of 26S rDNA partial sequences [8,14,26,27]. The results of NJ and ML phylogenetic trees based on the D1/D2 domain of 26S rDNA sequences were identical topologies according to Nitiyon *et al*. (2011) and Lorliam *et al*. (2013), and thus only the NJ tree is shown as Figure 3 [14,19]. It reveals that all strains isolated in this study were grouped in the *C. tropicalis* clade with the moderate supported value from NJ (77) and ML (83) methods. Therefore, we identified the Thai KPC strains isolated in this study as *C. tropicalis*. This species is known to have a worldwide distribution, especially in tropical and subtropical areas. There are several

thermotolerant yeasts which demonstrated as ethanol producing strain such as *S. cerevisiae*, *K. marxianus*, *Pichia* sp., and *Candida* sp. [1,7,9,10,12,19,21,26,28]. Although some reports have recently indicated that *C. tropicalis* YMEC14 expressing alpha-amylase and cellulose are driving the fermentation of starch to ethanol and *C. tropicalis* A26 isolated from cow faeces produced the highest xylitol concentration, the characterization of *C. tropicalis* which is applicable to industrial process has been less advanced [12,14]. In this study, it should be noticed that *C. tropicalis* KPC6 strain had relatively high potential to be used as alternative ethanol-producing yeast for ethanol production. Previously, Wu *et al*. (2016) reported that *K. marxianus* K21could produce ethanol at 48.98 g/L from taro waste at 40°C [29]. Lorliam *et al*. (2013) isolated *C. tropicalis* from cow faeces, and found that all *C. tropicalis* isolated produced ethanol 0.0016 - 0.0895 g/L after 24 h incubation under 30°C [14]. Buddiwong *et al*. (2014) isolated thermotolerant *C. tropicalis* strains, from sugarcane plantation of Thailand, which are able to produce maximum ethanol concentration at 52.55 g/L at 40°C [28]. Based on the ethanol production in our work, isolated *C. tropicalis* KPC6 had their ethanol fermentation ability at 42°C comparable to that of previously reports of isolated *C. tropicalis* strain. Further study on optimization of the fermentation factors for improving ethanol productivity of the strain KPC6 are currently proceeding.

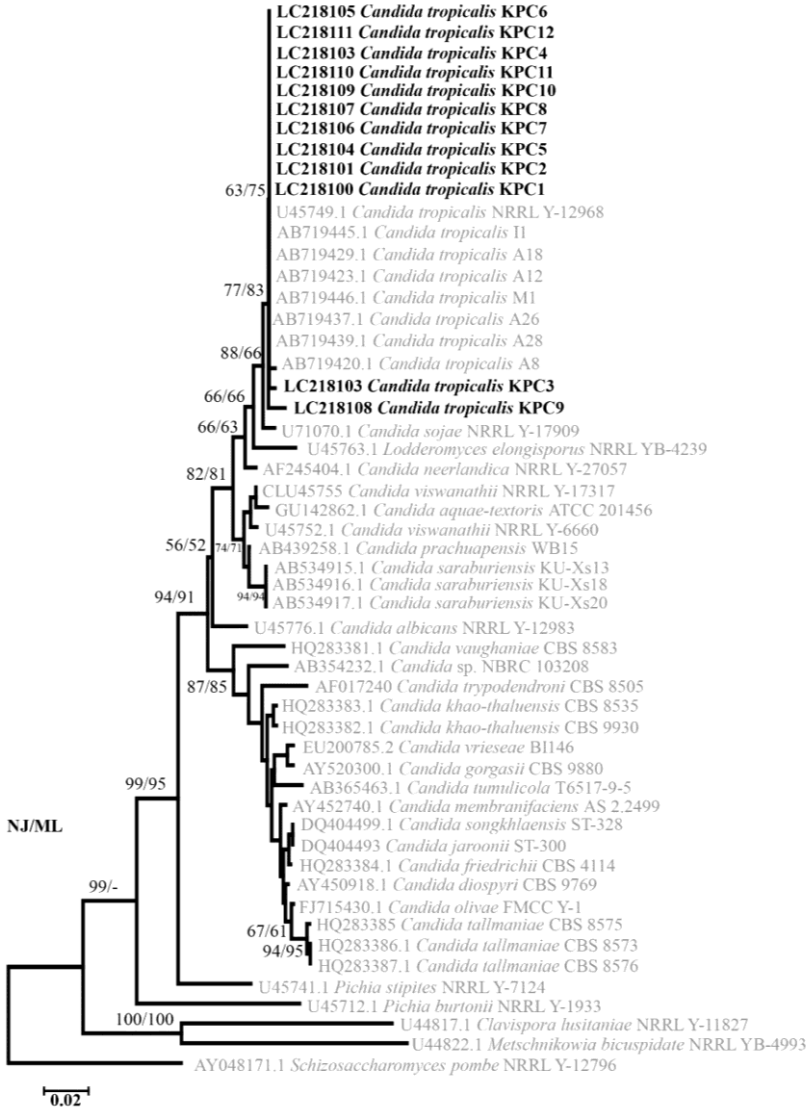

Figure 3 Phylogenetic tree of isolated strains constructed by the Neighbor-Joining method based on D1/D2 domain of LSU 26S rDNA sequences. The numbers represent the percentages from 1,000 replicates bootstrap resampling. *Schizosaccharomyces pombe* was used as an out-group.

4. Conclusion

Our findings allow us to conclude that *C. tropicalis* KPC strains isolated from Thai soil sugarcane plantation are not only tolerant to heat and ethanol stresses but also show a high specific growth rate value at higher temperature. The physiological characterization of these strains is important for enhancing the performance in

ethanol production and the KPC strains would be candidates for industrial and biological purposes, which would help to improve the productive efficiency of the biotechnological industry. However, there are several factors that affect the ethanol yields by microorganisms, such as pH levels of the medium, substrate concentration, carbon and nitrogen sources [30]. Further study on optimization of the fermentation factors for improving ethanol production are necessary.

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6. Conflict of Interest

No conflict of interest declared

7. References

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