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Screening of thermotolerant yeast isolated from sugarcane plantations in Northeastern part of Thailand

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Abstract

Isolation and screening of thermotolerant yeast capable of producing ethanol at high temperatures were described in this study. Soil, plant decay and sugarcane bagasse samples from sugarcane plantations and sugar factories in Udorn Thani, Khon Kaen, Nong Bue Lum Pu, Chaiyaphumi, Kalasil, Roi Ed and Nakorn Ratchasima province were collected and subjected to the isolation of thermotolerant yeast by using the enrichment culture technique. A total of 84 isolates of yeast were obtained and 43 isolates of them showed their ability to grow at temperature up to 45°C. Fifteen isolates of yeast could grow in the medium containing up to 10% (v/v) ethanol. Almost of the isolated yeasts utilized hexose sugars such as glucose, fructose and sucrose as carbon sources, while only 15 isolated yeasts utilized pentose sugars such as xylose and arabinose as carbon sources. Of 43 isolates of yeast, 19 isolates showed their ability to produce high concentration of ethanol at high temperatures. Molecular identification of the yeast based on the D1/D2 domain of 26s rDNA sequencing revealed that these 19 isolated yeasts belonged to the genus *Candida* sp., *Pichia* sp. and *Issatchenkia* sp.

Keywords: Biodiversity, Thermotolerant yeast, sugarcane plantation, ethanol production

1. Introduction

Ethanol fermentation at high temperatures using thermotolerant microorganisms has several advantages such as: a) reduction of cooling cost, b) decrease risk of contamination by mesophilic microorganisms, and c) increase rate of sugar uptake and ethanol fermentation (1). Recently, mesophilic ethanol-producing yeast, *Saccharomyces cerevisiae* has been widely used in a commercial ethanol production. However, the temperatures suitable for cell growth and ethanol production by this strain are relatively low (25-30°C). Previous studied by Kiran Sree et al. (2) reported that during ethanol fermentation temperature inside the bioreactor is raised from 30°C to approximately 40°C. This high temperature caused inhibition on cell growth as well as metabolic activity of yeast cell, resulted in reduction of ethanol yield and volumetric ethanol productivity. Therefore, seeking for

thermotolerant yeast species capable of producing ethanol at high temperatures is of great interested.Sugarcane is one of the economic crops widely grown in Thailand, particularly in the Northeastern part of the country where the average temperatures is relatively high throughout the year. The sugarcane plantation is expected to be one of the best ecosystems with greater diversity of microorganism especially ethanol-producing yeasts. Therefore, the aim of this study was to isolate and characterize the thermotolerant yeast from sugarcane plantations in the Northeastern part of Thailand. Growth property and ethanol production capability of the isolated yeasts under high temperature conditions were also described in the present study.

2. Materials and Methods

2.1 Sample collection

Soil, plant decay and sugarcane bagasse samples from sugarcane plantations and sugar factories in Udon Thani, Khon Kaen, Nong Bue Lum Pu, Chaiyaphumi, Kalasil, Roi Ed, Mahasarakham and Nakorn Ratchasima provinces were randomly collected and subjected to isolation of thermotolerant yeast.

2.2 Isolation of yeast

Isolation of yeast was carried out using the enrichment culture technique as described by Limtong et al. (3). One gram of samples was inoculated into 100-mL of autoclaved yeast extract-malt extract broth (YM broth) containing 0.3% yeast extract, 0.3% malt extract, 0.5% polypeptone and 1% glucose. The medium was also supplemented with 4% (v/v) ethanol. After inoculation, the culture broths were incubated in a shaker incubator (150 rpm) at 35°C for 3 days. 1% of the active culture was transferred into a fresh YM medium supplemented with 4% (v/v) ethanol and continuously culturing for 3 days as previously described. After 3 days of incubation, enriched cultures were streaked on YM agar plates and then incubated at 35°C. Colony appeared on the YM agar medium was collected and re-streaked until the pure cultures were obtained. All pure cultures were maintained on YM agar slants at 4°C and subculturing was performed every 3 months. For long-term storage, the cultures were kept in 50% (v/v) glycerol solution and stored at -20°C.

2.3 Screening of thermotolerant fermentative yeasts

2.3.1 Growth ability test at high temperatures

Thermotolerant yeasts were selected based on their growth ability at high temperatures (30 to 50°C) by streak plate technique, which was modified from that of Yuangsaard et al. (4). Each of the isolated strains was grown on YM agar medium and incubated at 30, 35, 37, 40, 45 and 50°C for 3 days. Growth ability of the isolated yeast strains was recorded and strains capable of growing at temperature higher than 37°C were selected for further tests.

2.3.2 Fermentation activity test at high temperatures

The fermentation activity test was carried out using the method described by Dung et al. (5). Briefly, each of the isolated strains was inoculated into 10-mL YM medium and incubated at 35°C for 24 h. A 0.1-mL of the active cultures was then transferred into 10-mL YM medium in a test tube containing a Durham tube and statically incubated at 37, 40 and 45°C for 3 days. Fermentation activity of the yeast was determined by monitoring the accumulation of CO_2 gas in the Durham tubes.

2.3.3 Ethanol tolerance capability test

An ability of selected thermotolerant yeasts to high ethanol concentrations (4-16% v/v) was tested based on the method described by Dung et al. (5). Each of the isolated yeast strains was grown on YM agar medium containing 4, 6, 8, 10, 12, 14 and 16% (v/v) ethanol and incubated at 30°C for 3 days. Growth of the isolated yeast strains on the YM agar medium was determined and compared.

2.3.4 Sugar utilization test

The sugar utilization ability of isolated yeasts was carried out using the method described by Lertwattanasakul et al. (6) with slightly modification. YM medium containing glucose, fructose, sucrose, xylose, arabinose at 1% (w/v) as carbon source was prepared and used in this study. Each of the isolated yeast strains was grown on YM medium containing different carbon sources as mentioned earlier and incubated at 30°C for 3 days. Growth of the isolated yeast strains on the medium was evaluated.

2.4 Ethanol production by thermotolerant fermentative yeast at high temperatures

The isolated yeast strains capable of growing at temperature above 37°C as well as tolerate to ethanol concentration at 10% (v/v) were selected and used to prepare seed cultures. Each isolate was inoculated into a test tube containing 10-mL YM liquid medium and incubated in a shaker incubator (150 rpm) at 35°C for 24 h. A 1-mL of the active cell was transferred into a 250-mL Erlenmeyer flask containing 100-mL YM liquid medium and then incubated in a shaker incubator (150 rpm) at 35°C to an early stationary growth phase (~9-12 h.). Cells were collected by centrifugation at 5,000 rpm for 5 min at 4°C, washed twice with sterile distilled water and resuspended in 0.85% NaCl. This active cell was inoculated into a 500-mL Erlenmeyer flask containing 300-mL YM medium containing 16% (w/v) glucose as carbon source. An initial cell concentration of the seed culture was $1x10^7$ cells/mL. The fermentation flasks were equipped with an air-lock and statically incubated at 37, 40 and 45°C for 72 h. A 5-mL fermentation broth was withdrawn from each flask every 12 h. and centrifuged at 13,000 rpm for 10 min at 4°C to remove yeast cells. The ethanol concentration (g/L) in the resulting supernatants was measured using gas chromatography (GC) (Shimadzu GC-14B, Japan) as described by Thanonkeo et al. (7).

2.5 Identification of yeast strains

Identification of the selected thermotolerant yeast strains was carried out using morphological and D1/D2 domain of 26s rDNA gene sequencing analysis (8). Yeast genomic DNA was prepared as described by Harju et al. (9). The D1/D2 domain of the 26s rDNA was amplified by PCR with specific primer NL-1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL-4 (5'-GGT CCG TGT TTC AAG ACG G-3') (10). After PCR amplification, the amplified product was separated by electrophoresis on 1.0% agarose gel, and purified using NucleoSpin® Extract II Kit (Machery-Nagel, Germany), according to the manufacture's instruction. Nucleotide sequence of the amplified product was determined by the ABI PRISM 310 sequencer (PE, Applied Biosystems) and homology analysis of the D1/D2 domain of 26s rDNA gene of selected thermotolerant yeast was performed using the homology search tool (blastn) (11). Phylogenetic tree was constructed using MEGA4, version 4.0 (12).

3. Results and Discussion

3.1 Sample collection and isolation of yeast

A total of 33 samples including soil, plant decay and sugarcane bagasses were collected from sugarcane plantations and sugar factories in Udon Thani, Khon Kaen, Nong Bue Lum Pu, Chaiyaphumi, Kalasil, Roi Ed, Mahasarakham and Nakorn Ratchasima provinces. A total of 84 isolates of yeast were derived after isolation using the enrichment culture technique (Table 1). Based on the number of isolated yeast, it can be concluded that sugarcane plantation is one of the best ecosystems for isolation of yeast.

3.2 Screening of thermotolerant fermentative yeast strains

All 84 isolates of yeast were tested for their growth ability at high temperatures (30-50°C) on YM agar medium. As the results showed in this study, about 43

Table 1. Sample collected for isolation of thermotolerant yeast and number of isolated yeast from different sources at different locations.

Location	Number of sample		Number of isolated yeast
	Soil	Bagasse	
Udon Thani	4	-	5
Khon Kaen	5	3	17
Nong Bue Lum Pu	1	-	2
Chaiyaphumi	1	-	2
Kalasil	7	1	28
Roi Ed	2	-	6
Mahasarakham	2	1	12
Nakorn Ratchasima	6	-	12
Total	28	5	84

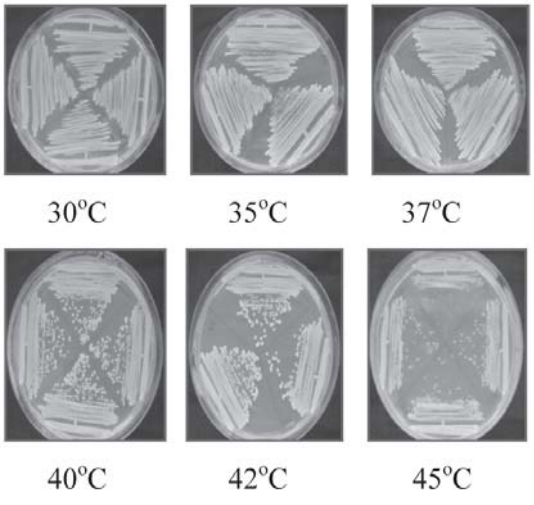

Figure 1. Growth of selected thermotolerant yeast on YM agar medium at high temperatures

isolates of them were capable of growing at temperature up to 45°C (Fig. 1). It was speculated from this finding that all these 43 isolated of yeast are thermotolerant yeasts, since they were able to grow at temperature above 37°C, as described by Lee et al. (13). All these 43 isolated of yeast were chosen for further analysis on the fermentation activity test at high temperatures. They were grown in 10-mL YM liquid medium in a test tube containing a Durham tube, then statically incubated at 37, 40 and 45°C for 3 days and the accumulation of CO_2 gas in the tube was recorded. The results showed that about 19 isolates of yeast, i.e., S1-2, S6-1, S10-1, S10-2, S11-2, S11-3, S15-1, S20-1, S23-5, S28-1, 28-2, S29-1, S29-2, S30-2, S30-3, S31-1, S32-1, S32-2 and S33-1, showed strong fermentation activity and produced CO_2 gas at 37, 40, and 45°C within 2 days after incubation (data not shown). Therefore, these isolates were selected for further tests.

The tolerance ability of isolated yeast to high concentration of ethanol was tested by culturing the yeast strains on YM agar medium containing 4, 6, 8, 10, 12, 14 and 16% (v/v) ethanol and incubated at 30°C. Among 43 isolates of selected yeast, 32 isolates were able to grown on the medium containing up to 8% (v/v) ethanol. Of 32 isolates of yeast, 15 isolates were able to grown on the medium containing up to 10% (v/v) ethanol concentration (data not shown). These results suggested that the isolated thermotolerant yeasts were not only tolerated to high temperatures but also high ethanol concentrations. This finding was in accordance to that of Chaudhuri et al. (14) who reported the relationship between the response of *Escherichia coli* cell to ethanol and heat stresses. Sugar utilization analysis of the isolated thermotolerant yeast was also performed. Yeast cells were grown on YM agar medium containing different carbon sources such as glucose, fructose, sucrose, xylose, arabinose at 1% (w/v) and incubated at 30°C. The results revealed that almost of the isolated yeast tested utilized glucose, fructose and sucrose as carbon sources. However, about 15 isolates of yeast showed their ability to grown on the medium containing xylose and arabinose as carbon sources (data not shown). Yeast species that able to assimilate and ferment both hexose and pentose sugars are found in various sources, e.g., rooten fruits (15), nectar of *Hibiscus R Osa-sinensis* and *Ixora coccinea* flowers (16). Based on the present results, sugarcane plantation is also one of a potential source of hexose- and pentose-utilizing yeast.

3.3 Ethanol production by selected thermotolerant yeast strains at high temperatures

Among the 43 isolates of thermotolerant yeast

obtained in this study, 19 isolates showed strong fermentation activity at high temperatures (37, 40 and 45°C), as measured by the accumulation of CO_2 gas in Durham tube. Thus, these isolates were subjected to the ethanol production tests by culturing the yeast cell in the YM medium containing 16% (w/v) glucose as described in Materials and Methods. At 37°C, the maximum ethanol concentration was found in the yeast isolate S10-2 (64.97 g/L), S6-1 (64.20 g/L), S11-3 (60.78 g/L) and S10-1 (60.00 g/L), respectively. The maximum ethanol concentration at 40°C was detected in the yeast isolate S10-2 (57.99 g/L), S11-3 (57.03 g/L), S6-1 (53.56 g/L) and S11-2 (52.55 g/L). When an incubation temperature was shifted to 45°C, the maximum ethanol concentration was detected in the yeast isolate S6-1 (37.43 g/L), S10-2 (37.09 g/L), S10-1 (36.27 g/L) and S1-2 (36.12 g/L) (Table 2). It can be seen from these results that the thermotolerant yeast isolate S10-2, S11-3 and S6-1 produced relatively high ethanol concentration at high temperatures tested (37°C, 40°C and 45°C), suggesting that these isolated yeasts had a high potential to be used as alternative ethanol-producing yeast for ethanol production in a commercial scale at high temperatures instead of the industrial used yeast, *S. cerevisiae*. Limtong et al. (3) isolated thermotolerant yeast from soil and water in sugarcane plantations and sugar factories in four provinces including Phra Nakhon Sri Ayutthaya, Ratchaburi, Suphanburi and Uthaithani, Thailand, and found one potential thermotolerant yeast namely *Kluyveromyces marxianus* DMKU 3-1042 which is able to produce ethanol with the concentration of 8.7% (w/v) and 6.78% (w/v) at 37°C and 40°C. Based on the ethanol concentration produced in this work, thermotolerant yeast isolated in this study, i.e., S10-2, S11-3 and S6-1, had their ethanol fermentation ability at high temperatures comparable to that of *K. marxianus* DMKU 3-1042.

Table 2 Ethanol production by selected thermotolerant yeast strains at different high temperatures.

Isolate	Maximum ethanol concentration (g/L)		
	37°C	40°C	45°C
S1-2	58.20^{g}	44.01^{h}	36.12^{d}
S6-1	64.20^{hi}	53.56^{ij}	37.43^{d}
S10-1	60.00^{g}	37.56^{f}	36.27^{d}
S10-2	64.97^{i}	57.99^{k}	37.09^{d}
S11-2	46.52^{e}	52.55^{i}	17.63^{ab}
S11-3	60.78^{gh}	57.03^{jk}	29.24^{c}
S15-1	34.00^{a}	27.75^{a}	16.46^{ab}
S20-1	42.30^{cde}	36.14^{ef}	17.01^{ab}
S23-5	51.03^{f}	35.73^{def}	19.66^{b}
S28-1	43.04^{cde}	30.24^{abc}	15.36^{ab}
S28-2	44.11^{de}	32.00^{bcd}	17.42^{ab}
S29-1	30.96^{a}	30.01^{abc}	14.46^{a}
S29-2	43.44^{cde}	33.66^{cde}	16.77^{ab}
S30-2	39.75^{bc}	30.57^{abc}	14.61^{a}
S30-3	37.88^{b}	33.02^{bcde}	18.33^{ab}
S31-1	39.46^{bc}	30.48^{abc}	14.77^{a}
S32-1	39.26^{bc}	29.20^{ab}	14.53^{a}
S32-2	33.77^{a}	31.79^{abcd}	17.32^{ab}
S33-1	40.67^{bcd}	31.91^{abcd}	15.47^{ab}

Mean values in each column having different letters are statically significantly different at $p<0.05$ based on Duncan Multiple Range Test (DMRT) analysis

3.4 Identification of selected thermotolerant yeast strains

A total of 19 isolates of thermotolerant yeast capable of producing ethanol at high temperatures were subjected to identification by D1/D2 domain of 26s rDNA sequencing analysis (8). Figure 2 showed phylogenetic analysis of nucleotide sequences of D1/D2 domain of 26s rDNA gene of selected thermotolerant yeast strains and those of related species from GenBank. Based on the phylogenetic analysis, the selected thermotolerant yeast strains can be clustered into 3 groups. The yeast isolate S1-2 and S10-2 were closely related to *Pichia kudriavzevii*, while isolate S6-1, S10-1 and S11-3 were closely related to *Issatchenkia orientalis* (synonym; *Candida krusei*). The last group, i.e., isolate S11-2, S15-1, S20-1, S23-5, S28-1, 28-2, S29-1, S29-2, S30-2, S30-3, S31-1, S32-1, S32-2 and S33-1 were closely related to *Candida tropicalis*, respectively.

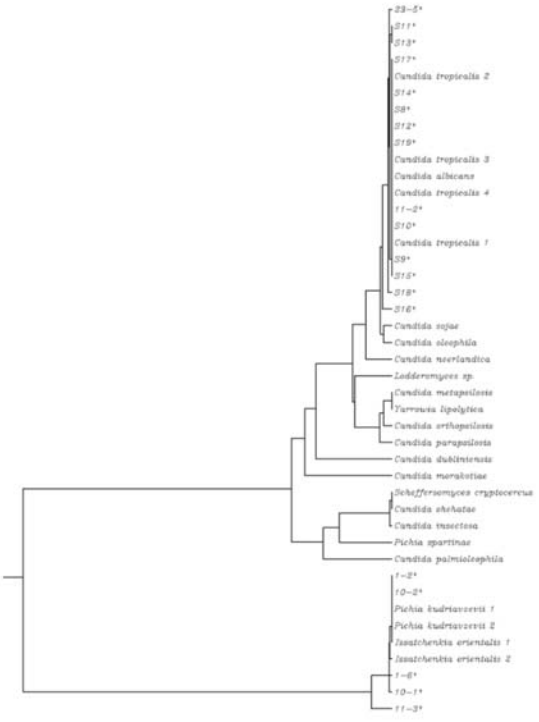

Figure 2. Phylogenetic tree showing the relationship between the isolated thermotolerant yeast strains and the related species in GenBank database.

There are several thermotolerant yeasts that have been previously reported as ethanol-producing strains, e.g., *K. marxianus* (3), *Pichia* sp., *Candida* sp., and some of *S. cerevisiae* (17). In this study, three species were found, i.e., *P. kudriavzevii*, *Iss. orientalis*, and *C. tropicalis*. It should be also noticed from this finding that *C. tropicalis* was a predominant thermotolerant yeast found in sugarcane plantation and sugar factories. The yeast *Candida* sp. has been found in various natural sources such as nectar of *Hibiscus R Osa-sinensis* and *Ixora coccinea* flowers (16) and maize (18)

4. Conclusion

Thermotolerant ethanol-producing yeast strains were successfully isolated in this study. Among the selected thermotolerant yeast strains tested, the newly isolated *P. kudriavzevii* S10-2 and *Iss. orientalis* S6-1 and S11-3 exhibited higher ethanol production capability at high temperature conditions than those of the other isolated yeast strains. The result demonstrated that all these three isolates were the effective strains that could be applied for ethanol production at elevated temperatures. The ethanol concentration produced by the selected thermotolerant yeast strains was relatively low as compared to those in the other works, since the ethanol fermentation in this study was carried out in flask scale without cultural optimization. To improve the ethanol yield produced by the selected thermotolerant yeast strains, cultural optimization using statistically experimental design should be performed both in the flask- and bioreactor-scale. Furthermore, application of the selected thermotolerant yeast strains for ethanol production from starch-based or lignocellulosic-based materials should also be carried out.

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