Isolation and selection of thermotolerant yeast for ethanol production at high temperature

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Abstract

Ethanol production at high temperatures not only increases the conversion rate of glucose to ethanol and reduces risk of contamination but also reduces the cost of cooling system and operating cost. In this study, thermotolerant ethanol-producing yeasts from different sampling sites; Chon Buri, Rayong, Chanthaburi, and Khon Kaen, Thailand were isolated. Based on the enrichment culture technique, a total of 127 yeast isolates were achieved. Forty isolates of them were able to grow at high temperatures of 37°C, 40°C and 45°C, and were chosen for further study on ethanol fermentation ability test. The yeast extract-malt extract (YM) medium containing 160 g/L glucose was used in this step, and the result showed that the yeast isolates Rz5-1, Rz3-1, Rz8-1, S6, S10 and S11 produced relatively high level of ethanol concentration at 37°C and 40°C as compared to the other isolates. Molecular identification of the yeast based on the D1/D2 domain of 26s rDNA sequencing revealed that all these isolates were *Pichia kudreiavzevii*. Since, *P. kudreiavzevii* Rz8-1 produced the highest ethanol concentration in the YM medium, thus it was chosen for further study on the ethanol production at high temperatures using the hydrolysate of sugarcane bagasse as substrate. The results found that at 37°C, *P. kudreiavzevii* Rz8-1 gave the maximum ethanol yield of 0.36 g/g substrate and the productivity of 1.47 g/L.h. At 40°C, this thermotolerant yeast produced the maximum ethanol yield of 0.34 g/g substrate and productivity of 1.39 g/L.h. Based on these results, *P. kudreiavzevii* Rz8-1 is a good candidate for ethanol production at high temperatures.

Keywords: *P. kudreiavzevii*, ethanol production, thermotolerant yeast, sugarcane bagasse

Introduction

High temperature fermentation (HTF) of ethanol is of great interest because it has several advantages such as; a) reduction of cooling cost, b) decrease risk of contamination by undesirable mesophilic microorganisms and c) increase rate of sugar to ethanol conversion, thereby it increases the ethanol productivity (Banat et al. 1998; Sootsuwan et al. 2007). Recently, many researchers have attempted to explore and characterize effective thermotolerant ethanol producing yeast strains that capable of growth and producing ethanol at high temperatures. There are several species of yeast that have been characterized and classified as thermotolerant yeast such as *Kluyveromyces marxianus*, *Pichia* sp., *Candida* sp., and some of the mesiphilic strain of *Saccharomyces cerevisiae* (Christensen et al. 2011; Limtong et al. 2007). Isono et al. (2012) reported an ability of thermotolerant yeast, *P. kudriavzevii* to grow and produce ethanol at temperature up to 43°C. In comparison with mesophilic yeast, *S. cerevisiae* which shows high ethanol fermentation efficiency and high

ethanol tolerance, *P. kudriavzevii* exhibits higher thermotolerant ability than that of *S. cerevisiae* (Chi and Arnebory, 2000; Edgardo et al. 2008).

Thermotolerant ethanol fermentative yeasts have been isolated from various natural sources. However, in this study we focus on the isolation of thermotolerant yeast from soil samples, plant bark decay, manure, rotten fruits collected in the plant orchards. Characterization of the selected thermotolerant yeast, such as high temperature tolerance, ethanol tolerance, as well as molecular identification of the selected thermotolerant yeast were described in this paper.

Methodology

Sample collection and isolation of yeast

Soil samples, plant bark decay, manure and rotten fruits from different orchards (Chon Buri, Rayong, Chanthaburi and Khon Kaen) were collected and subjected to isolation of yeast as described by (Yuangsaard et al. 2013) using YM medium supplemented with 4 % (v/v) ethanol. All pure colonies were stored in glycerol at -20^{o}C for long term storage.

Selection and characterization of thermotolerant yeast

Growth ability of isolated yeast at high temperatures was analyzed using the modified method described by Yuangsaard et al. (2013). The active cell of the isolated yeast was cultured in YM broth at 35^{o}C until its growth reached the early stationary phase (~9-12 h), then cells were subjected to serial dilution. 10 µL of the appropriated dilution culture was spotted onto YM agar plate and incubated at high temperatures (37, 40, 42 and 45^{o}C) for 1 day. Growth of isolated yeast on agar medium was recorded and compared to each other.

The ability of isolated yeast to withstand high ethanol concentration was also tested. Each of the isolated yeast was grown on YM agar medium supplemented with different ethanol concentrations (4, 6, 8, 10, 12 and 16 % (v/v)) and incubated at 35^{o}C for 3 days. Growth of isolated yeast was recorded.

The ethanol production by isolated yeast was evaluated by culturing each strain in 15 mL YM liquid medium in a test tube containing a Durham tube and then incubated at 37^{o}C for 72 h. The strains that capable of fermenting glucose and accumulating gas in the Durham tube were selected for further experiments.

Identification of selected thermotolerant yeast strains

Genomic DNA was isolated from selected thermotolerant yeasts using method described by Harju et al. (2004). The quality of the isolated genomic DNA was determined by agarose gel electrophoresis as well as spectrophotometry.

Identification of the selected thermotolerant yeast was carried out by molecular technique based on the ribosomal DNA sequencing analysis (Kurtzman and Robnett, 1998). The D1/D2 region of the large subunit, 26s rDNA gene, was amplified by PCR using specific primers NL-1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG) and NL-4 (5'-GGT CCG TGT TTC AAG ACG G). The amplified PCR product was separated on agarose gel electrophoresis and the target DNA fragment was excised from the gel and purified using NucleoSpin® Extract II kit (Machery-nagen, Germany), according to the manufacturer's instructions. Nucleotide sequence of the DNA 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). Phylogenetic tree was constructed based on the neighbor-joining method with 1000 bootstrap replicates using MEGA, version 5.0.

Ethanol fermentation at high temperature by selected thermotolerant yeast

The ethanol production efficiency at high temperatures of the selected thermotolerant yeast was evaluated in a 250 mL Erlenmeyer flask as described by Thanonkeo et al. (2007). The active cells were inoculated into 100 mL YM medium containing 16% glucose as carbon source and incubated on a rotary shaker (150 rpm) at 37, 40 and 45^{o}C. The ethanol concentration produced by each strain of selected thermotolerant yeast was measured using gas chromatography (GC) (Shimadzu GC-14B, Japan).

Ethanol production from hydrolysate of sugarcane bagasse by selected thermotolerant yeast

Sugarcane bagasse from sugar factory was treated with alkaline (2% NaOH) and acid (2% H_2SO_4) at 121^{o}C for 15 min. Then, it was treated with Cellic ® CTec2 (50 FPU/g DW) at 50^{o}C for 48 h. The resulting hydrolysate was collected and used as substrate for ethanol production by selected thermotolerant yeast.

Ethanol production from sugarcane hydrolysate was carried out in 250 mL Erlenmyer flask containing 100 mL of fermentative medium. After inoculation of the active yeast cells into the medium, flasks were incubated at high temperatures (37^{o}C and 40^{o}C) and samples were randomly collected and analyzed for ethanol concentration as mentioned above.

Results

Isolation of thermotolerant yeast

Based on an enrichment culture technique, a total of 127 isolated of yeast were achieved. Among of these isolates, 40 isolates were able to grow at temperature up to 40 and 45^{o}C (data not show). To select the thermotolerant fermentative yeast, all 40 isolates were grown in 15 mL YM broth in a test tube containing a Durham tube and their ability to ferment glucose and accumulated gas in the Durham tube were recorded. The results found that all 40 isolated of yeasts were able to ferment glucose and accumulated gas in the Durham tube within 24 h (data not shown). Thus, they were chosen for further tests.

Identification of thermotolerant yeast

All 40 thermotolerant yeast isolates were identified by D1/D2 domain of 26s rDNA gene sequencing. Based on the phylogenetic analysis as shown in Figure 1, five groups of yeast can be categorized. Nineteen isolated of yeast were closely related to *Candida tropicalis*, 15 were closely related to *Pichia kudriavzevii*, which was renamed from *Issatchenkia orientalis* (Kurtzman et al. 2011), 4 were closely related to *Candida grabrata* or *Candida albican*, 1 was closely related to *Candida orthopsilosis*, and the remaining was closely related to *Kodamea ohmeri*.

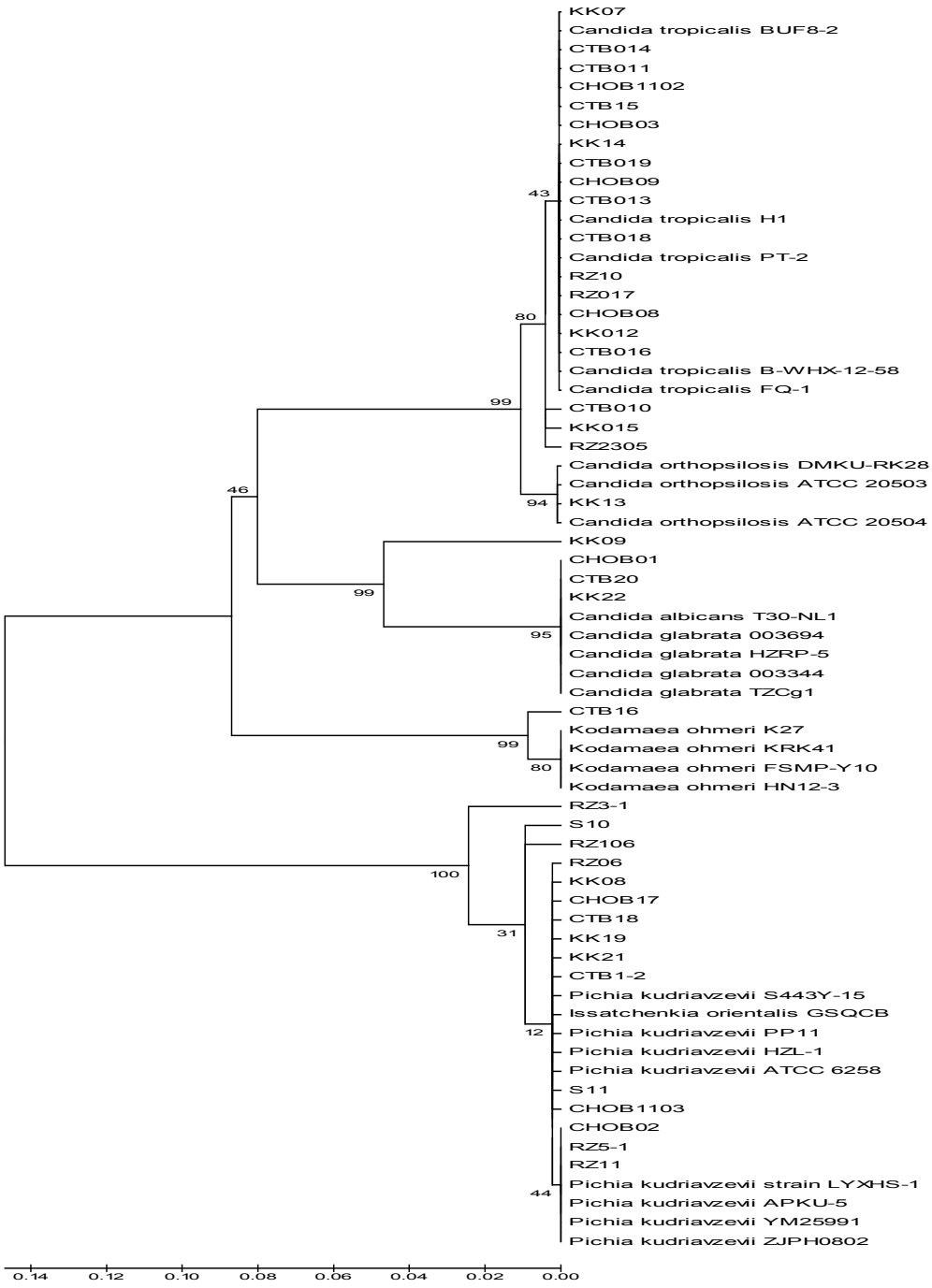

Figure 1: Neighbor-joining showing phylogenitic tree position of the isolated thermotolerant yeasts based on the nucleotide sequence of 26s rDNA gene.

Thermotolerance- and ethanol-tolerance ability of selected thermotolerant yeast

According to the preliminary test on ethanol fermentation ability of all 40 selected thermotolerant yeast in a test tube, 6 isolates of yeasts, namely Rz3-1, Rz5-1, Rz8-1, S6, S10 and S11, produced relatively high level of ethanol concentration as compared to other isolates tested. Therefore, these 6 isolates were chosen for further study on thermotolerance and ethanol tolerance ability test. As shown in Figure 2, all selected of yeasts were able to grow in the medium containing up to 10% ethanol. Nevertheless, only 3 isolated (RZ3-1, RZ5-1

and RZ8-1) exhibited their ability to grow at high temperature of 45°C as compared to other isolated.

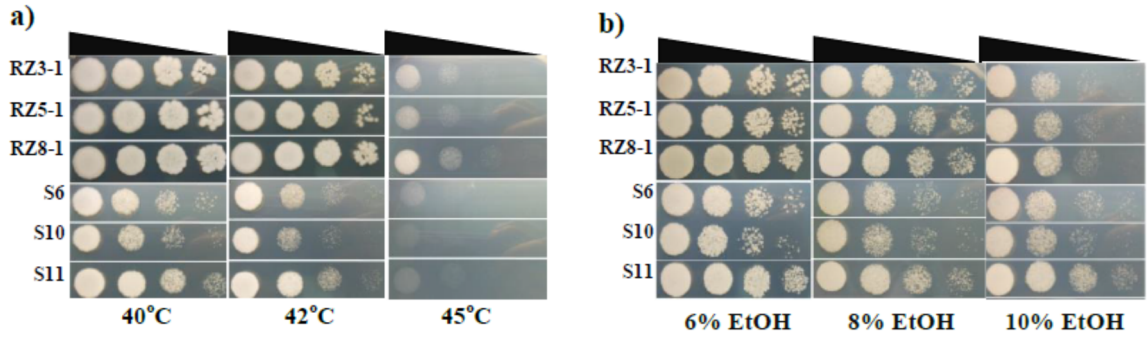

Figure 2: Growth of the selected thermotolerant yeasts at high temperatures (40, 42 and 45°C) (a) and high level of ethanol concentrations (6-10%) (b).

Ethanol production at high temperatures by selected thermotolerant yeast

The ethanol production by the selected thermotolerant yeast at high temperatures was determined and the results are summarized in Table 1. The results found that yeast strain RZ8-1 gave the highest ethanol concentrations at 37°C and 40°C while yeast strain RZ5-1 gave the highest yield at 45°C. Based on these results, yeast strain RZ8-1 was chosen for further study.

Table 1 Ethanol production by selected thermotolerant yeasts at high temperatures.

Isolates	Temperature (°C)	Time of fermentation (h)	Ethanol % (v/v)	Productivity (g/L.h)
RZ3-1	37	48	7.45±0.07	1.23
	40	48	6.78±0.09	1.12
	45	48	5.51±0.42	0.91
RZ5-1	37	48	7.79±0.14	1.29
	40	48	7.15±0.18	1.18
	45	48	5.68±0.03	0.94
RZ8-1	37	48	8.51±0.12	1.41
	40	48	7.50±0.79	1.24
	45	48	4.39±0.14	0.73
S6	37	48	8.08±0.17	1.34
	40	48	6.74±0.07	1.12
	45	48	4.71±0.21	0.78
S10	37	48	7.55±0.44	1.25
	40	48	6.54±0.01	1.08
	45	48	4.56±0.23	0.76
S11	37	48	8.18±0.25	1.35
	40	48	7.30±0.98	1.21
	45	48	4.67±0.14	0.77

Ethanol production from hydrolysate of sugarcane bagasse at high temperature by selected thermotolerant yeast

The chemical composition of the hydrolysate of sugarcane bagasse was analyzed and it was found that the hydrolysate contained approximately 97.6 g/L glucose, 25.4 g/L xylose and 1.7 g/L acetic acid (data not shown). When this hydrolysate was used as substrate for ethanol production at high temperatures by selected thermotolerant yeast, *P. kudriavzevii* Rz8-1, the ethanol yield produced by this thermotolerant yeast at 37°C was 0.36 g/g substrate and the maximum productivity was 1.47 g/L.h. At 40°C, the maximum ethanol yield and ethanol productivity were 0.34 g/g substrate and 1.39 g/L.h, respectively (data not shown).

Discussion

A total of 127 isolated of yeasts were achieved from samples collected from orchards in different locations. When all these isolated of yeasts were tested for their ability to grow at high temperatures, only 40 isolates were able to grow at temperature higher than 40°C. Based on the definition of thermotolerant yeast given by Kiran Sree et al. (2000), all these 40 isolates of yeast were expected to be thermotolerant yeast.

Identification of yeast can be carried out by biochemical test and molecular technique. The later method was the most promising approach as described by Kurtzman and Robnett (1998), Kurtzman et al. (2011). When all 40 isolates of yeast were subjected to identification by molecular technique using the D1/D2 domain of 26s rDNA sequencing, they can be classified into five groups, based on the phylogenetic analysis (Figure 1). These groups included *C. tropicalis* (19 isolates), *P. kudriavzevii* (15 isolates), *C. grabrata* or *C. albican* (1 isolate), *C. orthopsilosis* (1 isolate) and *K. ohmeri* (4 isolates). These findings indicated that orchard is a good habitat for the isolation of thermotolerant ethanol-producing yeast.

Only six isolates of yeast can produce relatively high level of ethanol concentration in the YM medium containing 160 g/L glucose. Among these strains, *P. kudriavzevii* Rz8-1 gave the highest ethanol content, as compared to the other strains tested. For the literature review, there is no reported described on ethanol production from hydrolysate of sugarcane bagasse by *P. kudriavzevii*. Thus, *P. kudriavzevii* Rz8-1 was chosen for ethanol production at high temperatures using the hydrolysate of sugarcane bagasse as raw material. As found in this study, the newly thermotolerant yeast, *P. kudriavzevii* Rz8-1 can produce relatively high ethanol yield (0.36 g/g substrate at 37°C and 0.34 g/g substrate at 40°C) and productivity (1.47 g/L.h at 37°C and 1.39 g/L.h at 40°C), which were higher than those reported by Kaur et al. (2012). These findings suggested that the newly thermotolerant yeast, *P. kudriavzevii* Rz8-1 is a good candidate for ethanol production from hydrolysate of sugarcane bagasse at high temperatures.

There are several factors that influence the ethanol production by microorganism, such as substrate concentration, nitrogen source, pH of the medium, etc. Therefore, further study on optimization of the fermentation factors should be carried out in order to improve the ethanol production efficiency, and this was under our investigation.

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