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High-temperature ethanol production using thermotolerant yeast newly isolated from Greater Mekong Subregion



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ABSTRACT

The application of high-potential thermotolerant yeasts is a key factor for successful ethanol production at high temperatures. Two hundred and thirty-four yeast isolates from Greater Mekong Subregion (GMS) countries, i.e., Thailand, The Lao People's Democratic Republic (Lao PDR) and Vietnam were obtained. Five thermotolerant yeasts, designated *Saccharomyces cerevisiae* KKU-VN8, KKU-VN20, and KKU-VN27, *Pichia kudriavzevii* KKU-TH33 and *P. kudriavzevii* KKU-TH43, demonstrated high temperature and ethanol tolerance levels up to 45 °C and 13% (v/v), respectively. All five strains produced higher ethanol concentrations and exhibited greater productivities and yields than the industrial strain *S. cerevisiae* TISTR5606 during high-temperature fermentation at 40 °C and 43 °C. *S. cerevisiae* KKU-VN8 demonstrated the best performance for ethanol production from glucose at 37 °C with an ethanol concentration of 72.69 g/L, a productivity of 1.59 g/L/h and a theoretical ethanol yield of 86.27%. The optimal conditions for ethanol production of *S. cerevisiae* KKU-VN8 from sweet sorghum juice (SSJ) at 40 °C were achieved using the Box–Behnken experimental design (BBD). The maximal ethanol concentration obtained during fermentation was 89.32 g/L, with a productivity of 2.48 g/L/h and a theoretical ethanol yield of 96.32%. Thus, the newly isolated thermotolerant *S. cerevisiae* KKU-VN8 exhibits a great potential for commercial-scale ethanol production in the future.

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Introduction

At present, the world is facing an energy crisis caused by the continuous use of fossil oil. As a result, petroleum oil use has dropped sharply.¹ Bioethanol is an alternative energy

source of particular interest whose production by microbial fermentation is increasing to replace gasoline.^{2–4} Yeasts, particularly *Saccharomyces* spp., are the most common ethanol producers employed in industry.⁵ Yeast has proven to be more effective for ethanol production than bacteria due to

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its good fermentative capacity and its ability to tolerate high concentrations of ethanol and the by-products formed during pretreatment and fermentation. *S. cerevisiae* is capable of fermenting different types of sugars, such as glucose, fructose and sucrose to ethanol via the glycolysis pathway under anaerobic conditions.^{6,7} The optimal growth temperatures of yeasts range from 25 to 35 °C. However, heat stress as well as other stresses such as ethanol and osmotic pressure which are generated during fermentation process, greatly affect ethanol production and decrease the specific growth rate of yeast strains.^{8,9}

Many types of raw materials can be used as potential substrates for ethanol production. They are classified into the following three major groups: (1) sugars such as sugarcane, sugar beet, sweet sorghum, whey and molasses^{3,4,10,11}; (2) starches such as corn, wheat, cassava and potato^{12–15}; and (3) lignocellulosic feedstock such as woody materials, agricultural wastes and crop residues.^{16–18} In recent years, interest in the utilization of sweet sorghum juice (SSJ) for ethanol production has increased in the southern United States, India, China and other countries including Thailand. The advantageous attributes of SSJ include a shorter growing period for sweet sorghum, lower requirements for fertilizers and water, and lower cultivation costs compared to sugarcane.^{10,11,19,20}

Ethanol production at high temperatures has garnered much interest due to several advantages, including reduced cooling costs and a reduced risk of contaminations.²¹ To achieve high-temperature fermentation, the fermentation capability of yeasts and its ability to grow and produce ethanol under a variety of inhibitory conditions must be considered, particularly at elevated temperatures and the accumulation of high concentrations of ethanol. Many studies have examined various thermotolerant yeasts and raw materials for use in ethanol production under optimized conditions. *Pichia kudriavzevii* grows at 42 °C and produces ethanol concentrations in the range of 29–78.6 g/L from sugarcane and cassava starch hydrolysate at 40 °C.^{4,15,17,22} Other yeasts, such as *S. cerevisiae* IR2 and IR2*, *S. cerevisiae* VS1, VS3 and *Kluyveromyces marxianus* DMKU 3-1042, grow at higher temperatures between 42 °C and 45 °C and achieve ethanol concentrations at 40 °C varying from 28 to 67.8 g/L when sugarcane and lignocellulosic biomass are used as substrates.^{3,5,8} Although yeasts can grow at elevated temperatures above 40 °C, the optimal temperature for ethanol production is approximately 30 °C.^{6,10,12,23}

SSJ contains an abundant carbon source and certain minerals essential for yeast growth and ethanol production¹⁰ but lacks certain other inorganic constituents, vitamins and biogenic elements, potentially limiting ethanol fermentation under stresses such as high-temperature conditions.²⁴ Several researchers have reported the addition of exogenous nutrients, such as ammonium sulfate, magnesium sulfate, potassium dihydrogen phosphate, manganese sulfate or yeast extract to increase fermentation efficiency and ethanol yield.^{3,15,24–28} Recently, statistically designed experiments have been widely employed to optimize ethanol production conditions.^{24,26,28} These techniques provide several advantages, such as reductions in consumption time and operating costs due to the use of fewer experimental units and the evaluation of interactions between independent variables. Furthermore, optimal conditions are determined with a

second-order polynomial equation.²⁹ Many factors, including incubation temperature, initial yeast cell concentration, initial pH and sugar concentration, affect the growth and ethanol production of yeast cells.^{3,11,26,30}

Given the benefits of yeast thermotolerance, which can significantly reduce ethanol production costs, the search for thermotolerant yeasts is a major challenge in achieving high ethanol production levels. There is little information regarding thermotolerant yeast species and their distribution in the three countries of the Greater Mekong Subregion (GMS) (Thailand, Lao PDR and Vietnam) as well as their potential in ethanol production from SSJ at high temperatures. In addition, microbial populations from different geographic locations likely exhibit differential thermotolerance and fermentation capabilities. Therefore, the aims of this study were to isolate and identify thermotolerant and ethanol-tolerant yeast strains from specific regions of Thailand, Lao PDR and Vietnam. We identified five thermotolerant yeast strains, of which two were *P. kudriavzevii* and three were *S. cerevisiae*, exhibiting unique characteristics in ethanol production experiments employing YM medium and SSJ as substrates. The optimal conditions for ethanol production from SSJ by one of the newly isolated thermotolerant yeast strains were also investigated using the Box–Behnken experimental design (BBD). Finally, we described the ethanol production potential of the yeast strain using SSJ under high-temperature fermentation conditions.

Materials and methods

Sample collection

The samples used in this study were collected from fruits, flowers and other sources, such as banana, papaya, grape, orange, apple, mango, Vietnamese apple flowers, longan flowers, papaya flowers, alcoholic beverages, soil and sawdust, from Thailand, Lao PDR and Vietnam. One gram (or 1 mL if the sample was a liquid) of a sample was added to 100 mL of yeast extract-malt extract (YM) medium (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 20 g/L glucose) and incubated at 35 °C for 24 h. One percent (v/v) inoculum was added into YM liquid medium containing 4% (v/v) ethanol and incubated at 35 °C, which were employed as selective pressures for obtaining thermotolerant yeasts, for 24 h.³ Then, a spread plate technique was used to obtain isolated colonies on YM agar containing 4% (v/v) ethanol at 35 °C incubation temperature for 24 h. A single colony of each isolate was selected and streaked on YM agar containing an ethanol concentration of 4% (v/v) and incubated at 35 °C for 24 h. Samples were kept at –20 °C for further analysis.

Screening and isolation of thermotolerant and ethanol-tolerant yeasts

Thermotolerant yeast isolates were selected based on their growth performance at 37 °C, 40 °C and 45 °C on YM agar. Isolated yeasts capable of growing at 40 °C or higher were selected for further screening and identification. To screen for ethanol-tolerant yeasts, the thermotolerant yeast isolates were inoculated into YM medium at 35 °C for 24 h. An initial

cell concentration of 1×10^6 cells/mL was inoculated into YM medium containing different ethanol concentrations of 7, 10 and 13% (v/v). Then, the cultures were incubated at 35 °C for 48 h. Viable yeast cell numbers were determined by the direct counting method using a haemocytometer and the methylene blue staining technique.³¹ Ethanol tolerance was determined based on the percentage of survival, which could be divided into three categories: highly tolerant (>50% survival), moderately tolerant (25–50% survival) and slightly tolerant (<25% survival).³²

Identification of selected thermotolerant yeasts

Identification of the selected thermotolerant yeasts was performed using morphological characterization and molecular identification. For morphological characterization, the physical appearance of the selected thermotolerant yeasts was observed according to the classical methods described by Barnett et al.³³ The cell morphology was examined under bright field microscopy (Carl Zeiss Primo star microscope, Carl Zeiss microscopy, Germany). Molecular identification was carried out by performing sequencing analysis of the D1/D2 domain and the internal transcribed spacer (ITS) 1 and 2 regions. DNA was extracted from yeast cells using a protocol described by Harju et al.³⁴

The D1/D2 domain of the 26S rDNA was amplified by polymerase chain reaction (PCR) using the primer pairs NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3').³⁵ The internal transcribed spacer (ITS) 1 and 2 regions were also amplified by PCR using the primer pairs ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3').³⁶ The amplification conditions consisted of initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 1 min, annealing at 53 °C for 1 min, and extension at 72 °C for 1 min; and a final extension at 72 °C for 7 min. The PCR products were analyzed by agarose gel electrophoresis and sequenced at First Base Laboratories (First BASE Laboratories Sdn Bhd, Malaysia). The amplified D1/D2 and ITS sequences of the yeast isolates were compared to the sequences of related species retrieved from the National Center for Biotechnology Information (NCBI) database using BLASTN. Phylogenetic analysis was performed using the neighbor-joining method with the Molecular Evolutionary Genetics Analysis (MEGA) program version 5.2. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were also determined.³⁷

Preparation of raw material for ethanol production

Sweet sorghum cv. KKU40 was kindly provided by the Faculty of Agriculture, Khon Kaen University, Thailand, and was used as a substrate for ethanol production in this study. The fresh juice was extracted from sorghum stalks by squeezing in a juice extractor; then, the juice was concentrated to 65°Bx via evaporation and maintained at –20 °C prior to use. The composition of SSJ was analyzed at the Central Laboratory (Thailand) Co., Ltd. in Thailand (Table 1).

Table 1 – The composition of sweet sorghum juice cv. KKU40.

Composition	Content
pH	4.90
Total sugar (g/L)	143.95
Sucrose ^a (g/L)	108.20
Glucose ^a (g/L)	21.80
Fructose ^a (g/L)	13.30
Protein ^b (mg/L)	1,900.00
Total N ^b (mg/L)	300.00
P ^c (mg/L)	247.25
K ^c (mg/L)	3,447.00
Ca ^c (mg/L)	418.20
B ^c (mg/L)	0.61
Co ^c (mg/L)	<0.01
Cu ^c (mg/L)	Not detected
Fe ^c (mg/L)	3.76
Mg ^c (mg/L)	147.10
Mn ^c (mg/L)	1.06
Mo ^c (mg/L)	0.02
Ni ^c (mg/L)	0.05
Na ^c (mg/L)	51.78
S ^c (mg/L)	Not detected
Zn ^c (mg/L)	0.46

The composition of SSJ was analyzed at the Central Laboratory, Co., Ltd., Thailand.

^a HPLC.

^b AOAC (2012), 981.10.

^c Atomic absorption.

Ethanol production by selected thermotolerant yeasts using glucose as a substrate

The ethanol production efficiency of the thermotolerant yeast strains was tested in 250-mL Erlenmeyer flasks containing 100 mL of YM broth and 200 g/L of glucose. The YM medium was inoculated with thermotolerant yeast strains at an initial yeast cell concentration of approximately 1×10^7 cells/mL and incubated at 37 °C or 40 °C for 48 h. Viable yeast cell numbers were determined by the direct counting method using a haemocytometer and the methylene blue staining technique.³¹ The fermentation broth was centrifuged at 13,000 rpm for 10 min, and the total residual sugar concentration of the supernatant was tested using the phenol sulfuric acid method.³⁸ Ethanol production was analyzed by gas chromatography as described by Laopaiboon et al.¹¹ The ethanol yield ($Y_{p/s}$) was calculated based on the actual ethanol produced and expressed as g of ethanol per g of sugar utilized (g/g). The ethanol productivity (Q_p , g/L/h) was calculated from the final ethanol concentration per the fermentation time at the highest ethanol concentration. The isolated yeast strains with ethanol concentrations greater than 35 g/L were chosen for further study.⁸

Ethanol production by selected thermotolerant yeasts using SSJ as a substrate

Sterile SSJ containing 200 g/L of total sugar was inoculated with the selected thermotolerant yeast strains at an initial yeast cell concentration of approximately 1×10^7 cells/mL. *S. cerevisiae* TISTR 5606, an industrial strain used

for commercial-scale ethanol production in Thailand, was used as a reference strain. The culture was incubated with shaking at 150 rpm at 37 °C, 40 °C or 43 °C for 48 h. After 48 h of incubation, viable yeast cell numbers, the total residual sugar concentration and ethanol production were analyzed as previously mentioned. Thermotolerant yeast strains exhibiting ethanol yields similar to that of *S. cerevisiae* TISTR 5606 were selected for ethanol production at 37 °C, 40 °C and 43 °C for 72 h. During ethanol fermentation, samples were collected every 6 h, and viable yeast cell numbers, the total residual sugar concentrations and ethanol production were analyzed according to previously described protocols.

Optimization of ethanol fermentation conditions using statistical experimental design

To identify the significant variables affecting ethanol production from SSJ at a high temperature (40 °C) by the newly isolated thermotolerant *S. cerevisiae* KKU-VN8 strain, various fermentation factors which were selected based on a literature review, including initial sugar concentration, initial pH, ammonium sulfate ((NH₄)₂SO₄) and trace elements such as magnesium sulfate (MgSO₄·7H₂O) and manganese sulfate (MnSO₄·H₂O), were evaluated using the Plackett–Burman design. The following two levels (low and high) for each experimental variable were employed: A (initial sugar concentration, g/L): 180 and 250; B (initial pH): 4 and 6; C ((NH₄)₂SO₄, g/L): 1 and 5; D (MgSO₄·7H₂O, g/L): 0.1 and 0.5; and E (MnSO₄·H₂O, g/L): 0.02 and 0.1.

Fermentation factors exerting a significant effect on ethanol production efficiency at 40 °C by *S. cerevisiae* KKU-VN8 were evaluated based on the Plackett–Burman design. These significant factors were then selected for optimization using the Box–Behnken design (BBD). In this study, three variables, specifically initial sugar concentration, initial pH and (NH₄)₂SO₄ concentration, were chosen, and ethanol concentration was selected as a response variable. According to the BBD, three levels corresponding to the low, middle and high values for each experimental variable were employed: A (initial sugar concentration, g/L): 150, 195 and 240; B (initial pH): 4, 5 and 6; and C ((NH₄)₂SO₄, g/L): 0.1, 1.55 and 3. The Design-Expert 7.0 demo version (STAT EASE Inc., Minneapolis, USA) was used to generate the experimental designs and perform regression analysis. Analysis of Variance (ANOVA) was carried out to estimate statistically significant parameters. The

quality of the quadratic model equation was expressed as the coefficient of determination (R^2). A validation experiment was conducted using the optimized conditions determined from the response surface plots. The experiments were carried out in 500-mL Erlenmeyer flasks incubated on a rotary shaker at 150 rpm and 40 °C for 72 h. During ethanol fermentation, samples were withdrawn, and total residual sugars and ethanol production were analyzed according to previously described protocols.

Effects of the initial cell concentration of *S. cerevisiae* KKU-VN8 on ethanol production efficiency using SSJ as a substrate

Effects of the initial cell concentration of *S. cerevisiae* KKU-VN8 on ethanol production from SSJ were investigated. One hundred milliliters of SSJ containing the optimized factors, i.e., 238.52 g/L of initial total sugars, 2.04 g/L of ammonium sulfate and the pH value of 5.82 was inoculated with different initial yeast cell concentrations of 1×10^7 , 1×10^8 and 3×10^8 cells/mL, and incubated on a rotating shaker at 150 rpm at 40 °C for 48 h. Samples were taken out every 12 h. The viable yeast cell number, the total residual sugar concentration and the ethanol concentration were analyzed according to previously described protocols. Acetic acid and glycerol concentrations were analyzed by high performance liquid chromatography (HPLC) using an Aminex HPX-87H column (Bio-Rad Laboratories, CA, USA) and refractometer detection at 40 °C. The samples were eluted with 5 mM sulfuric acid at a flow rate of 0.6 mL/min.

Results

Screening and isolation of thermotolerant and ethanol-tolerant yeasts

Two hundred and thirty-four yeast isolates were screened and isolated from Thailand, Lao PDR and Vietnam. Twenty-six promising isolates, specifically KKU-TH1, KKU-TH33, KKU-TH43, KKU-TH54, KKU-TH57, KKU-TH105, KKU-TH199, KKU-TH200 and KKU-TH211 isolated from the northeastern region of Thailand, KKU-LA1, KKU-LA4, KKU-LA9, KKU-L14, KKU-L22, KKU-L26 and KKU-L28 isolated from Vientiane city in Lao PDR and KKU-VN7, KKU-VN8, KKU-VN9, KKU-VN20, KKU-VN25, KKU-VN27, KKU-VN28, KKU-VN30, KKU-VN35

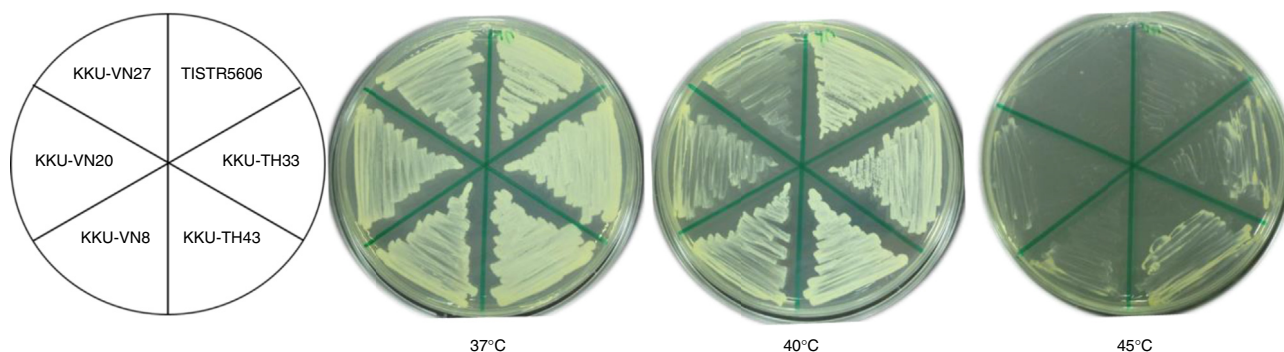


Fig. 1 – Growth performance of thermotolerant yeast isolates at 37, 40 and 45 °C after 24-h incubation on YM plates.

and KKU-VN36 isolated from Can Tho city in Vietnam, were selected for preliminary thermotolerance and ethanol tolerance studies. Fig. 1 shows the growth of selected thermotolerant yeast isolates at 37 °C, 40 °C and 45 °C. All isolates grew well at 37 °C. Seven isolates from Thailand, six isolates from Lao PDR and two isolates from Vietnam demonstrated good growth at 40 °C (data not shown). Only five isolates, specifically KKU-TH33, KKU-TH43, KKU-TH199, KKU-LA1 and KKU-LA4, demonstrated moderate growth at 45 °C, while others exhibited weak or no growth (Fig. 1).

To test for ethanol tolerance, the selected thermotolerant yeast isolates were inoculated into YM medium containing 7, 10 and 13% (v/v) ethanol.³⁹ The majority of the isolates tolerated ethanol concentrations up to 10% (v/v) (Fig. 2). Interestingly, the isolates KKU-TH33, KKU-TH43, KKU-TH200, KKU-TH211 and KKU-VN35 were highly tolerant of high ethanol concentrations up to 13% (v/v).

Identification of selected thermotolerant yeasts

The selected thermotolerant yeast colonies exhibited similar physical appearances on YM agar. Colonies were cream-white in color and circular in shape. They possessed a convex profile and were smooth and opaque with a glossy surface. Microscopic characteristics of the selected yeasts included spherical and egg shapes with budding, either isolated or grouped (data not shown). Molecular analysis by PCR showed that the PCR products obtained using the NL1 and NL4 primers consisted of bands of approximately 600 bp. However, the PCR products obtained using the ITS1 and ITS4 primers were of different sizes, ranging from 500 to 900 bp (data not shown). Phylogenetic relationships were determined by comparing the sequences of the ITS1 and ITS2 regions available in the GenBank sequence database to those of the isolated thermotolerant yeasts (Fig. 3). Twenty-six promising isolates demonstrated sequence identities of 99%. The isolates KKU-TH1, KKU-TH57 and KKU-TH105 were identified as *Candida tropicalis*. The isolates KKU-TH33, KKU-TH43, KKU-TH54, KKU-TH199, KKU-TH200, KKU-LA1, KKU-LA4, KKU-LA9, KKU-LA14, KKU-LA22, KKU-LA26, KKU-LA28 and KKU-VN36 were identified as *P. kudriavzevii*. The isolates KKU-VN8, KKU-VN9, KKU-VN20, KKU-VN25 and KKU-VN27 were identified as *S. cerevisiae*. The isolates KKU-TH211 and KKU-VN7 were identified as *Torulaspota globosa* and *C. glabrata*, respectively. All twenty-six thermotolerant yeast strains were employed for subsequent ethanol production experiments.

Ethanol production by selected thermotolerant yeasts using glucose as a substrate

The ethanol production efficiency of the selected thermotolerant yeast strains in YM medium containing 200 g/L of glucose was examined. After incubation at 37 °C for 48 h, all twenty-six strains produced ethanol at concentrations in the range of 37.47–72.69 g/L, productivities of 0.78–1.59 g/L/h and ethanol yields of 0.27–0.44 g/g (Fig. 4A). *S. cerevisiae* KKU-VN8 exhibited the best performance for ethanol production at 37 °C, with an ethanol concentration of 72.69 g/L, productivity of 1.59 g/L/h and an ethanol yield of 0.44 g/g. All strains with the exception of KKU-VN30, KKU-VN35 and KKU-VN36 were chosen for

subsequent ethanol production experiments at 40 °C, owing to their low yields and ethanol concentrations.

Ethanol production at 40 °C identified twenty-three strains demonstrating differential ethanol production, with ethanol concentrations ranging from 16.52 to 53.89 g/L, productivities of 0.34–1.12 g/L/h and ethanol yields of 0.22–0.44 g/g (Fig. 4A). *S. cerevisiae* KKU-VN8, KKU-VN20, KKU-VN27, *P. kudriavzevii* KKU-LA28, KKU-TH199 and *T. globosa* KKU-TH211 produced high ethanol concentrations of 53.89, 50.62, 50.53, 44.68, 43.27 and 43.21 g/L, respectively. Based on the criteria described by Sree et al.,⁸ yeast strains capable of producing ethanol concentrations and yields greater than 35 g/L and 0.33 g/g, respectively, were selected for further experiments. Therefore, *T. globosa* KKU-TH211, *P. kudriavzevii* KKU-TH33, KKU-TH43, KKU-TH199, KKU-LA1, KKU-LA4, KKU-LA28, *S. cerevisiae* KKU-VN7, KKU-VN8, KKU-VN20 and KKU-VN27 were employed to investigate ethanol production using SSJ as a substrate.

Ethanol production by selected thermotolerant yeasts using SSJ as a substrate

Eleven thermotolerant yeasts and *S. cerevisiae* TISTR 5606 as a reference strain were inoculated into SSJ containing 200 g/L total sugar and incubated at 37 °C with an agitation rate of 150 rpm for 48 h. The selected thermotolerant yeast strains produced ethanol concentrations in the range of 11.94–47.39 g/L with productivities of 0.25–0.99 g/L/h and ethanol yields of 0.13–0.45 g/g (Fig. 4B). Although the values were lower than those of reference strain, eight potential strains, specifically *T. globosa* KKU-TH211, *P. kudriavzevii* KKU-TH33, KKU-TH43, KKU-TH199, KKU-LA1, *S. cerevisiae* KKU-VN8, KKU-VN20 and *S. cerevisiae* KKU-VN27, were further investigated for ethanol production at 40 °C based on their ethanol yields, which were similar to that of the reference strain.

Ethanol fermentation at 40 °C by these eight strains resulted in ethanol concentrations that varied from 25.20 to 48.46 g/L, with productivities and yields of 0.52–1.01 g/L/h and 0.31–0.43 g/g, respectively. In terms of ethanol concentrations, productivities and yields of the five strains, specifically *P. kudriavzevii* KKU-TH33 and KKU-TH43, which were isolated from Thailand, and *S. cerevisiae* KKU-VN8, KKU-VN20, and KKU-VN27, which were isolated from Vietnam, performed better than the reference strain at 40 °C (Fig. 4B). Thus, they were selected to investigate ethanol production at 43 °C. These five promising strains, particularly *S. cerevisiae* KKU-VN8, KKU-VN20 and KKU-VN27, produced much higher ethanol concentrations than that of the reference strain at 40 °C and 43 °C, although a minute decrease in ethanol concentration was observed at elevated temperatures. When ethanol production performance of the reference strain was evaluated at 40 °C and 43 °C, ethanol concentrations substantially decreased, suggesting a lower degree of thermal tolerance than the five promising strains.

Batch culture profiles of the selected thermotolerant yeasts, specifically *P. kudriavzevii* KKU-TH33, and KKU-TH43, *S. cerevisiae* KKU-VN8, KKU-VN20, and KKU-VN27 and the reference strain *S. cerevisiae* TISTR 5606, using SSJ at 37 °C, 40 °C and 43 °C for 72 h were investigated. Ethanol concentration continued to increase during fermentation over 72 h at 37 °C.

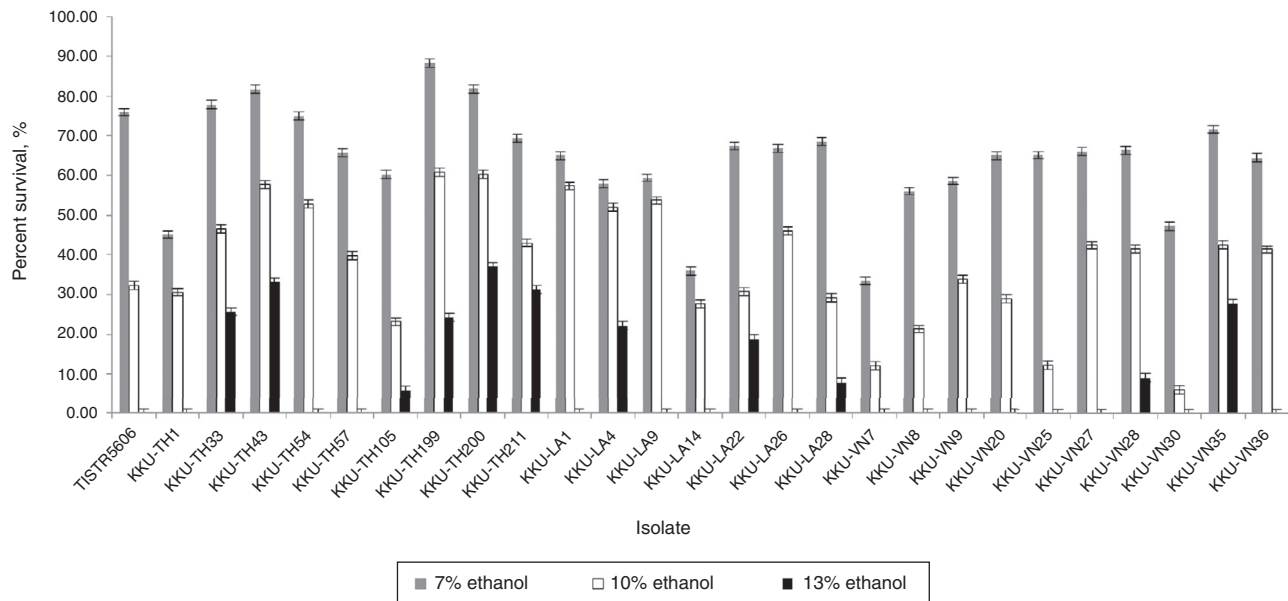


Fig. 2 – The percentage of survivals of thermotolerant yeast isolates in YM medium with different ethanol concentrations (7, 10 and 13% (v/v)) after 48 h.

Although the ethanol concentrations and yields of *S. cerevisiae* KKU-VN8, KKU-VN20 and KKU-VN27 and the reference strain were approximately the same at 48 h of fermentation, *S. cerevisiae* KKU-VN8, KKU-VN20 and KKU-VN27 demonstrated much greater values when the fermentation time reached 72 h (Fig. 5). Because the ethanol production of the reference strain was stable at 48 h, the fermentation parameters of all strains were compared during this time period (Table 2). At 40 °C and 43 °C, ethanol concentrations, productivities and yields of all five strains were much higher than those of the reference strain (Table 2, Fig. 5).

Optimization of ethanol fermentation conditions in statistically designed experiments

The significance of each variable for ethanol production by *S. cerevisiae* KKU-VN8 was determined using the Plackett-Burman design implemented with Design Expert

software. The experimental design matrices as well as the response variable were evaluated. The ethanol concentrations measured in 12 experiments ranged from 41.87 to 57.76 g/L (Additional information section, Table 1). To identify significant variables affecting ethanol production, a Pareto chart of standardized effects was constructed (Fig. 6). Based on the t-value limit, three variables, specifically initial sugar concentration (A), initial pH (B) and (NH₄)₂SO₄ concentration (C), were considered significant variables; variables A and C exerted negative effects on ethanol production, while variable B exerted a positive effect.

The statistical significances of the variables and the established model were confirmed by ANOVA (Table 3). In this study, the established model was highly significant because the p-value of the experiment was less than 0.05; thus, the model is reliable. As shown in Table 3, variables A, B and C exerted significant effects on ethanol production from SSJ, as

Table 2 – Ethanol production efficiency of thermotolerant yeast strains at 37, 40 and 43 °C for 48 h using SSJ.

Strain	Temperature (°C)								
	37			40			43		
	P (g/L)	Q _P (g/L/h)	Y _{P/s}	P (g/L)	Q _P (g/L/h)	Y _{P/s}	P (g/L)	Q _P (g/L/h)	Y _{P/s}
<i>S. cerevisiae</i> TISTR5606	49.44 ± 0.16 ^d	1.03 ± 0.00 ^d	0.42	34.35 ± 0.31 ^a	0.72 ± 0.01 ^a	0.40	34.31 ± 0.50 ^a	0.71 ± 0.01 ^a	0.40
<i>P. kudriavzevii</i> (KKU-TH33)	42.60 ± 0.14 ^b	0.89 ± 0.00 ^b	0.40	41.82 ± 0.49 ^c	0.87 ± 0.01 ^c	0.40	40.08 ± 0.21 ^c	0.83 ± 0.00 ^c	0.42
<i>P. kudriavzevii</i> (KKU-TH43)	40.64 ± 0.21 ^a	0.75 ± 0.01 ^a	0.39	38.65 ± 0.41 ^b	0.81 ± 0.01 ^b	0.39	37.49 ± 0.50 ^b	0.78 ± 0.01 ^b	0.40
<i>S. cerevisiae</i> (KKU-VN8)	49.64 ± 0.36 ^d	1.03 ± 0.01 ^d	0.45	48.51 ± 0.37 ^e	1.01 ± 0.01 ^e	0.43	43.52 ± 0.32 ^e	0.91 ± 0.01 ^e	0.42
<i>S. cerevisiae</i> (KKU-VN20)	48.58 ± 0.03 ^c	1.01 ± 0.00 ^c	0.44	47.59 ± 0.49 ^{de}	0.99 ± 0.01 ^d	0.43	42.57 ± 0.24 ^d	0.89 ± 0.00 ^d	0.41
<i>S. cerevisiae</i> (KKU-VN27)	48.09 ± 0.06 ^c	1.00 ± 0.00 ^c	0.44	46.89 ± 0.49 ^d	0.98 ± 0.01 ^d	0.42	40.39 ± 0.33 ^c	0.84 ± 0.01 ^c	0.39

P, ethanol concentration (g/L); Q_P, volumetric ethanol productivity (g/L/h); Y_{P/s}, ethanol yield (g/g).

^{abcde} values bearing different superscripts within the same column are significantly different using Duncan's multiple range test at the level of 0.05. The results were expressed as mean ± SD.

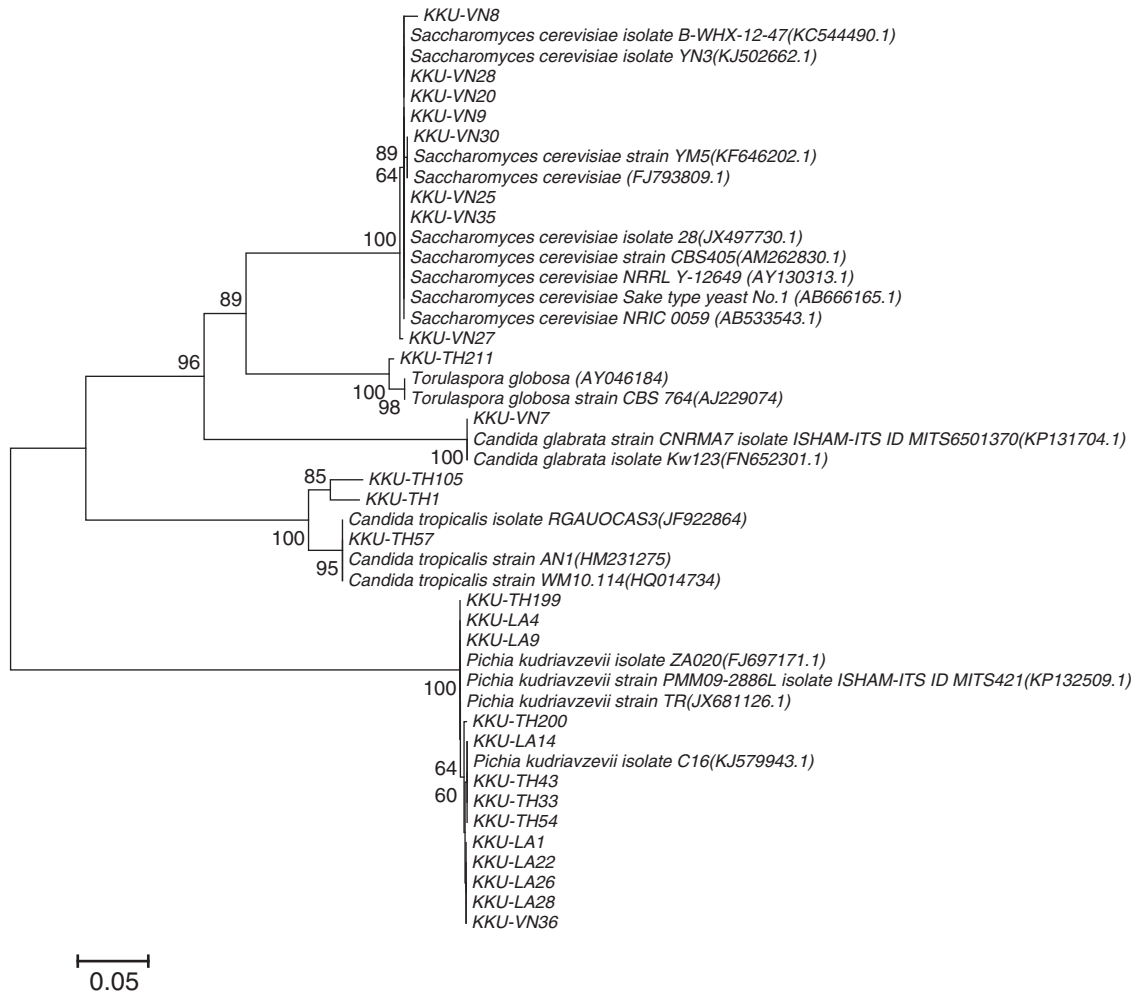


Fig. 3 – Phylogenetic relationships of yeast strains and the related taxa based on a combined sequence analysis of the internal transcribed spacer (ITS) 1 and 2 regions. Clustering was performed by the neighbor-joining using maximum likelihood with the software package, Molecular Evolutionary Genetics Analysis (MEGA) version 5.2. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. A scale bar of 0.05 is scale of branch lengths of the evolutionary distances used to infer the phylogenetic tree.

demonstrated in the Pareto chart analysis. Therefore, these three variables were chosen for optimization experiments using the Box–Behnken design to identify the best combination of variables resulting in maximum ethanol production efficiency.

The experimental design matrices and the ethanol concentration (g/L), as the response variable, were evaluated. Ethanol

concentrations measured in 16 experiments ranged from 31.18 to 59.10 g/L (Additional information section, Table 2). To develop a quadratic polynomial regression model and a second-order polynomial equation (1) to predict the final ethanol concentration (P, g/L) as a function of the fermentation variables, including the initial sugar concentration (A), the initial pH (B) and the (NH₄)₂SO₄ concentration (C), the ethanol

Table 3 – Analysis of variance for selected factorial model for ethanol concentration.

Source	Sum of squares	df	Mean square	F value	p-value Prob > F	
Model	201.84	5	40.37	7.48	0.0147	significant
A-Sugar	72.82	1	72.82	13.49	0.0104	
B-pH	71.65	1	71.65	13.28	0.0108	
C-NH	46.69	1	46.69	8.65	0.0259	
D-Mg	2.02	1	2.02	0.37	0.5629	
E-Mn	8.66	1	8.66	1.60	0.2522	
Residual	32.38	6	5.40			
Cor Total	234.22	11				

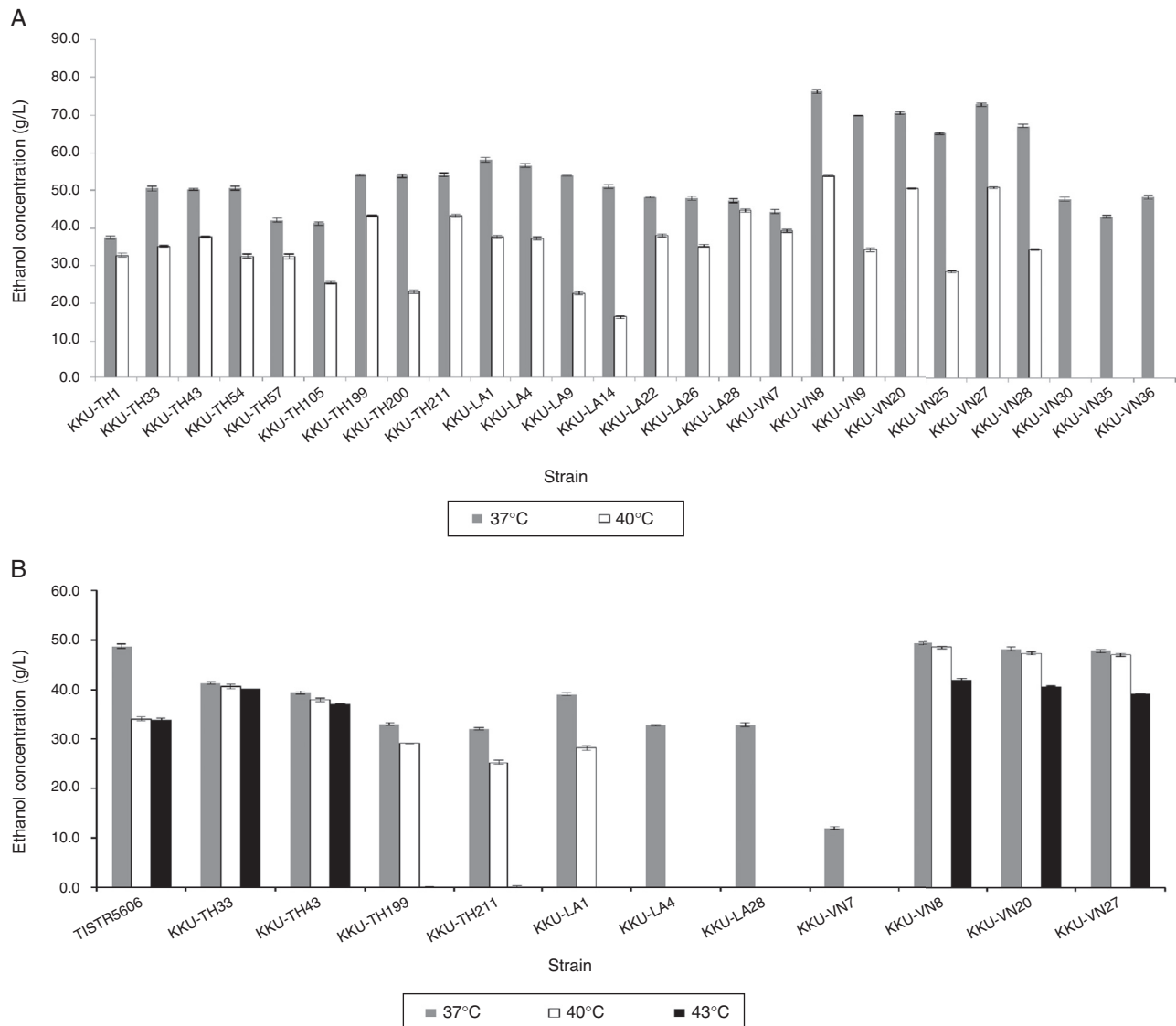


Fig. 4 – Ethanol production from thermotolerant yeast strains using YM medium (A) or SSJ (B) containing 200 g/L of total sugars with pH adjustment to 4.5 at 37 °C (gray), 40 °C (white) and 43 °C (black) for 48 h.

concentrations were used, and the prediction equation was as follows:

$$P(\text{g/L}) = 53.52 + 2.75A + 3.76B + 10.15C - 0.44AB - 1.55AC + 2.55BC + 1.04A^2 - 1.91B^2 - 9.02C^2 \quad (1)$$

The statistical significance of the model was determined by ANOVA (Table 4). The established model was highly significant (p -value less than 0.05). In addition, the linear terms of the initial sugar concentration (A), initial pH (B), and $(\text{NH}_4)_2\text{SO}_4$ concentration (C), the interactions between each variable (AB, AC and BC) and the quadratic terms of each variable (A^2 , B^2 and C^2) were also significant. The R^2 value of the regression (0.9997) suggested that 99.97% of the variability in the response was explained by this model. The p -value obtained from the “lack-of-fit tests” (0.1757) was not significant (p -value

greater than 0.05). Based on these results, the model is clearly reliable.

3-D response surface plots illustrating the effects of all selected variables on ethanol production by *S. cerevisiae* KKU-VN8 at 40 °C are shown in Fig. 7. Fig. 7A shows the effects of the initial sugar concentration and the initial pH on ethanol concentration. Ethanol concentration increased when the levels of both variables increased. In addition, ethanol concentration gradually increased when levels of the initial sugar concentration and the $(\text{NH}_4)_2\text{SO}_4$ concentration increased (Fig. 7B). Fig. 7C shows the effects of initial pH and $(\text{NH}_4)_2\text{SO}_4$ concentration on the ethanol concentration when the initial sugar concentration is fixed at the center point. According to the results, the ethanol concentration increased when the initial pH and the $(\text{NH}_4)_2\text{SO}_4$ concentration increased to a certain level.

To verify the predicted optimal values, repeated ethanol production experiments utilizing SSJ at 40 °C with *S. cerevisiae*

Table 4 – Analysis of variance for response surface of quadratic model for the Box–Behnken design.

Source	Sum of squares	df	Mean square	F value	p-value Prob > F	
Model	1378.02	9	153.11	2166.35	<0.0001	Significant
A-Sugar	60.37	1	60.37	854.13	<0.0001	
B-pH	112.96	1	112.96	1598.29	<0.0001	
C-NH	823.78	1	823.78	11655.34	<0.0001	
AB	0.79	1	0.79	11.16	0.0156	
AC	9.65	1	9.65	136.52	<0.0001	
BC	25.92	1	25.92	366.80	<0.0001	
A ²	4.36	1	4.36	61.65	0.0002	
B ²	14.57	1	14.57	206.18	<0.0001	
C ²	325.62	1	325.62	4607.05	<0.0001	
Residual	0.42	6	0.071			
Lack of fit	0.33	3	0.11	3.31	0.1757	Not significant
Pure error	0.098	3	0.033			
Cor total	1378.45	15				

R-Squared (R²) = 0.9997; Adj R-Squared = 0.9992.

Table 5 – Ethanol production from SSJ by *S. cerevisiae* KKU-VN8 at 40 °C for 36 h.

Initial cell concentration (cells/mL)	P (g/L)	Q _P (g/L/h)	T.Y. (%)	Acetic acid (g/L)	Glycerol (g/L)
1 × 10 ⁷	64.79 ± 0.21	1.80 ± 0.01	91.15	0.67 ± 0.00	14.52 ± 0.01
1 × 10 ⁸	69.97 ± 0.32	1.94 ± 0.01	92.77	0.96 ± 0.00	13.19 ± 0.01
3 × 10 ⁸	89.32 ± 0.25	2.48 ± 0.01	96.32	1.57 ± 0.00	12.53 ± 0.08

P, ethanol concentration (g/L); Q_P, volumetric ethanol productivity (g/L/h); T.Y., theoretical yield of ethanol (%).
The results were expressed as mean ± SD.

KKU-VN8 were performed. The following final optimal values of the selected variables from the repeated experiments were achieved: initial sugar concentration of 238.52 g/L, initial pH of 5.82 and (NH₄)₂SO₄ concentration of 2.04 g/L. The maximum ethanol concentration (65.67 g/L), volumetric ethanol productivity (1.37 g/L/h) and theoretical ethanol yield (93.24%) were achieved under the optimal conditions and were reliably similar to the predicted values (data not shown).

Effects of the initial cell concentration of *S. cerevisiae* KKU-VN8 on ethanol production efficiency using SSJ as a substrate

Since 49.64% of the remaining sugars were observed in the medium after 48 h of the optimized ethanol fermentation, it was plausible that increasing the initial cell concentration correlated with increased ethanol production. Thus, the experiments varying the initial cell concentration from 1 × 10⁷ cells/mL to 3 × 10⁸ cells/mL were conducted. Effects of the initial cell concentrations on ethanol production at 40 °C using SSJ under optimized conditions are summarized in Table 5. By increasing the initial yeast cell concentrations from 1 × 10⁷ cells/mL to 3 × 10⁸ cells/mL, ethanol production after the fermentation time of 36 h greatly increased. It was demonstrated that the maximal ethanol concentration of 89.32 g/L with the productivity and the theoretical ethanol yield of 2.48 g/L/h and 96.32%, respectively, were achieved when the initial cell concentration of 3 × 10⁸ cells/mL was used (Table 5). The by-products, i.e., acetic acid and glycerol were quantified after 36 h of fermentation. Acetic acid and

glycerol concentrations of 1.57 and 12.53 g/L, respectively, were observed (Table 5).

Discussion

Heat and ethanol stresses induce functionally overlapping stress responses in yeast cells. Yeasts respond to heat and ethanol stresses by synthesizing heat-shock proteins following exposure to temperatures above 35 °C or ethanol levels greater than 4–6% (v/v).⁴⁰ The combination of a temperature of 35 °C and an ethanol concentration of 4% (v/v) is suitable and chosen to screen for thermotolerant yeasts.³ If a yeast is able to grow at a temperature slightly above the maximum temperature (T_{max}) of mesophilic yeast, which ranges from 35 to 40 °C, it is considered thermotolerant.⁴¹ Thus, the results from this study indicated that putative thermotolerant yeasts were obtained. Many studies have reported thermotolerant yeasts growing at temperature ranges similar to those described in this study; however, differential ethanol production was observed.^{8,12,15,17,42} The differences in ethanol production might be due to differences in natural sources for the isolation and strains of thermotolerant yeasts, in which this study differs from those reported by Limtong et al.,³ Yuangsard et al.,¹⁵ Kwon et al.,¹⁷ Kaewkrajay et al.,⁴³ and Charoensoparat et al.⁴⁴

A high ethanol concentration above 10% (v/v) negatively affects yeast growth because it inhibits cell division and cell viability, resulting in a decreased specific growth rate or increased cell death.^{45,46} Therefore, yeasts that tolerate

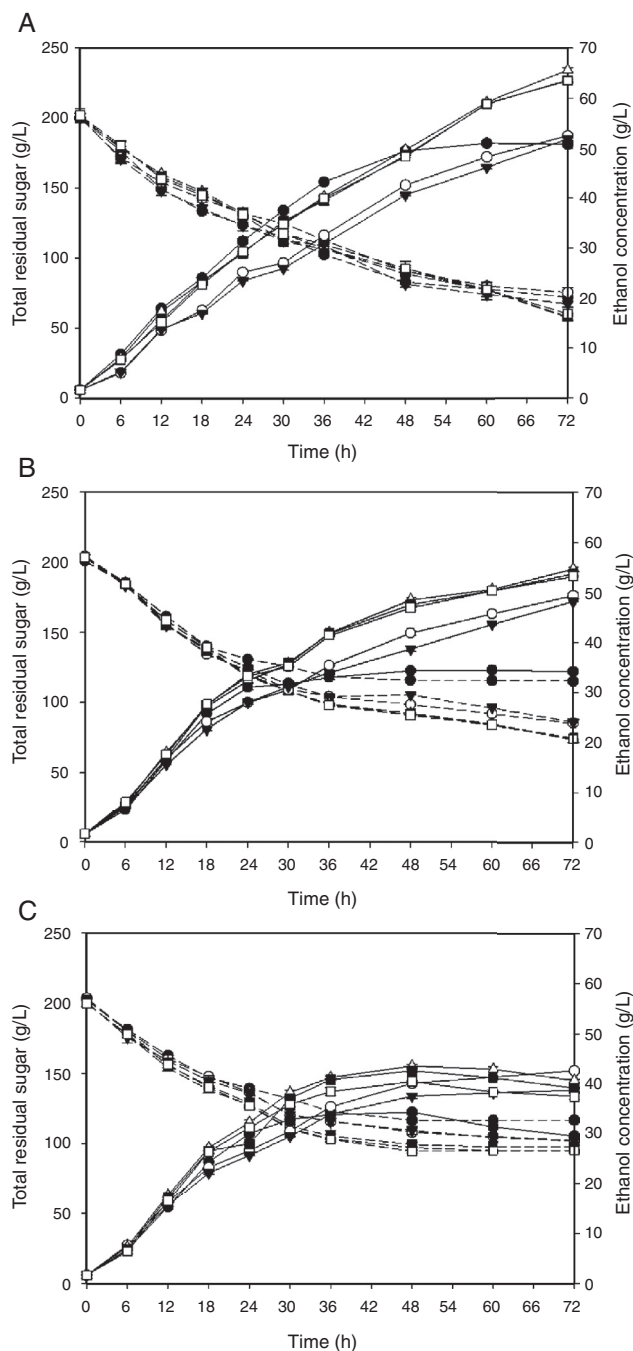


Fig. 5 – Batch culture profiles of ethanol production from SSJ at the initial sugar concentration of 200 g/L with pH 4.5 at 37 °C (A), 40 °C (B) or 43 °C (C) by *S. cerevisiae* TISTR 5606 (●), *P. kudriavzevii* KKU-TH33 (○), *P. kudriavzevii* KKU-TH43 (▼), *S. cerevisiae* KKU-VN8 (△), *S. cerevisiae* KKU-VN20 (■) and *S. cerevisiae* KKU-VN27 (□): total residual sugars (dash line), ethanol concentration (solid line). The results were expressed as mean ± SD.

stress conditions, particularly ethanol stress, are required for ethanol production. The results obtained in our study in terms of ethanol tolerance were consistent with those of Negi et al.,³² and Tikka et al.,³⁹ who found that *S. cerevisiae* strain N and strain YMI, respectively, tolerated ethanol concentrations up

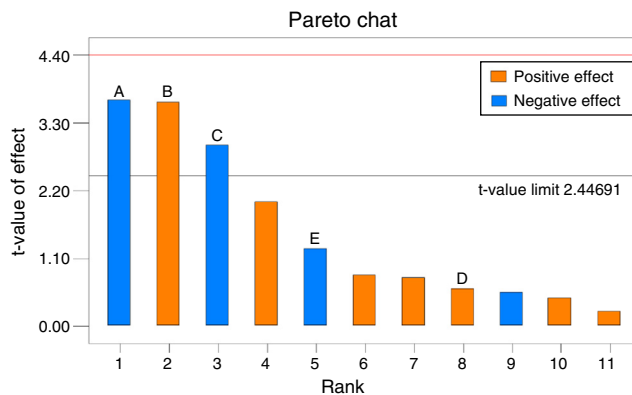


Fig. 6 – Pareto chart of standardized effects for a Plackett-Burman design of initial sugar concentration (A); initial pH (B); $(\text{NH}_4)_2\text{SO}_4$ concentration (C); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ concentration (D); and $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ concentration (E).

to 12% (v/v). However, the mutant *S. cerevisiae* UVNR56 tolerated higher ethanol concentrations than those observed in this study because *S. cerevisiae* UVNR56 was treated with UV-C radiation, which potentially modified the genes involved in certain stress-tolerant mechanisms such that ethanol tolerance was improved to 15% (v/v).⁴⁷

High temperatures and high ethanol concentrations are major stress factors for yeast during the fermentation process, inhibiting cell division and the specific growth rate.⁴⁸ In addition, these factors affect various transport systems such as the general amino acid permease and glucose uptake processes.^{5,49} The yeast isolates obtained in our study, which possess high temperature and ethanol tolerance levels, potentially develop both short-term and long-term stress response mechanisms to cope with the deleterious effects of these stresses, including the synthesis of heat shock proteins (HSPs) and trehalose as well as alterations to the lipid composition of the plasma membrane.⁵⁰⁻⁵³

The observed physical appearances and microscopic characteristics of the yeasts were in good agreement with the results of other investigators.^{12,42} For molecular and phylogenetic analyses, both ITS regions and D1/D2 domain were employed to identify thermotolerant yeasts at species levels. According to Chen et al.,⁵⁴ the ITS1 and ITS2 regions are useful to differentiate closely related species, particularly those with identical D1/D2 domain DNA sequences. In addition, these regions distinguish yeast strains exhibiting differences of 10 bp or more in terms of PCR product lengths.⁵⁵ Thus, species identification accuracy should depend on both the D1/D2 domain of the large subunit of 26S rDNA as well as the ITS1 and ITS2 regions. There are five yeast species identified in this study, i.e., *C. tropicalis*, *P. kudriavzevii*, *S. cerevisiae*, *T. globosa* and *C. glabrata*. Although *C. tropicalis* was previously reported as a leukemia-causing medical pathogen, it is also a candidate for ethanol production from olive prunings⁵⁶ and rice straw hydrolysate.⁵⁷ *P. kudriavzevii* was previously reported to produce ethanol from sugarcane juice,⁴ cornstalk hydrolysate¹⁷ and cassava hydrolysate.¹⁵ *S. cerevisiae* is frequently employed and demonstrates high potential in industrial ethanol production. Substrates utilized for ethanol production by *S. cerevisiae*

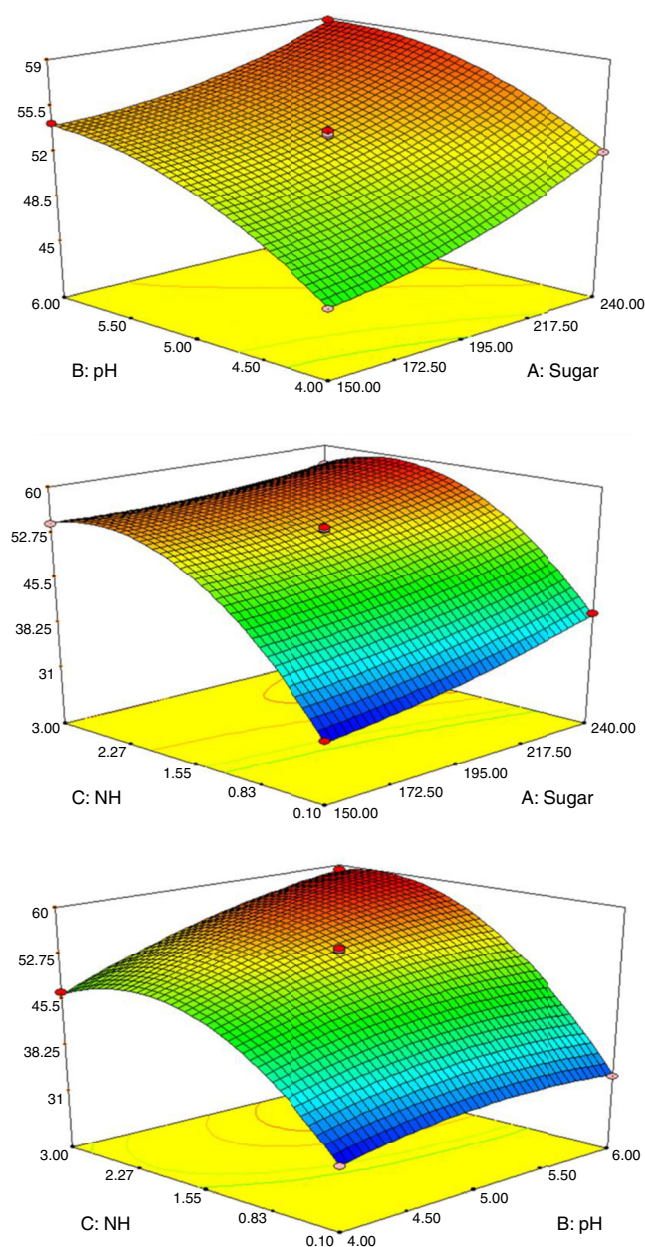


Fig. 7 – Response surface plots of effects of the initial sugar concentration and the initial pH (A), the initial sugar concentration and the ammonium sulfate concentration (B) and the initial pH and the ammonium sulfate concentration (C) on efficiency of ethanol production. Increase in the initial sugar concentration, the initial pH and the ammonium sulfate concentration resulted in varying ethanol concentrations from 31.18 g/L (blue) to 59.10 g/L (red).

are cassava powder hydrolysate,¹² wheat straw hydrolysate¹⁸ and SSJ.¹⁰ *T. globosa* is reported to be utilized for the biological control of fungal growth,⁵⁸ oil production for biodiesel^{59,60} and ethanol production.⁶¹ However, *C. glabrata* is reported to be a medical pathogen.^{62,63}

The ethanol concentrations produced by yeast strains obtained in our study were greater than those reported by

Sree et al.,⁸ when using glucose as a substrate under the same fermentation conditions at 40 °C. The same trend was clearly observed when SSJ was used as a substrate for ethanol production. The promising strains, particularly *S. cerevisiae* KKU-VN8, produced much higher ethanol concentrations than that of the reference strain as well as those reported by Sree et al.,⁸ Tomás-Pejó et al.,⁶⁴ and Kaewkrajay et al.,⁴³ at 40 °C and 43 °C. These results may be attributable to the higher levels of stress tolerance exhibited by the potential strains during fermentation, leading to better ethanol production and the unique stress-tolerant mechanisms, allowing the yeasts cope with environmental stresses during ethanol fermentation, including the synthesis of Hsps and trehalose.^{50–53} Moreover, the batch culture profiles showed that ethanol production and sugar consumption decreased as temperature increased, potentially because some sugars were converted to by-products such as glycerol, succinate, acetic acid and fusel oil.⁶ Yeast cells are affected by various stress factors, including nutrient deficiency, high temperature, organic acids, osmotic pressure and ethanol stress.⁹ These stresses inhibit not only yeast growth and viability but also affect other metabolic processes. To cope with these problems, Hsps, trehalose and glycogen metabolism are induced to protect yeast cells from these stresses.^{50,51,53,65}

Significant variables and their interactions as well as the response variables were determined with the reliable model and validated. The Box–Behnken design was employed and demonstrated the best combination of variables, resulting in maximal ethanol production efficiency. A comparative analysis of the ethanol production from SSJ by the newly isolated thermotolerant *S. cerevisiae* KKU-VN8 strain and other raw materials used in combination with different yeast strains reported in the literature is shown in Table 6. The ethanol concentration produced by *S. cerevisiae* KKU-VN8 was higher than that of *K. marxianus* DMKU 3-1042 using sugar cane juice as a substrate,³ *Issatchenkia orientalis* IPE100¹⁷ and *S. cerevisiae* AT-3⁶⁶ using glucose, *P. kudriavzevii* DMKU 3-ET15 using cassava starch hydrolysate,¹⁵ *S. cerevisiae* NRRL Y-2034,⁶⁷ *S. cerevisiae* DBKKU Y-53³⁰ and *S. cerevisiae* SEMF1²⁸ using SSJ. Although the percentage of sugar consumption by *S. cerevisiae* KKU-VN8 was lower than those of Zhao et al.,²⁸ Nuanpeng et al.,³⁰ it exhibited a substantially greater theoretical ethanol yield than those previously reported (Table 6). The differences in ethanol production efficiency are potentially attributable to differences in raw materials, yeast strains and initial cell concentration used for fermentation. In particular, the determination of the appropriate initial cell concentration is essential for the improvement of substrate utilization and ethanol yield, as typically observed in industrial ethanol fermentation.⁷ Charoensopharat et al.,⁴⁴ Nuanpeng et al.,³⁰ and Laopaiboon et al.¹⁰ reported that increasing the initial cell concentration improved the ethanol concentration and productivity. Our study also demonstrated that a high initial cell concentration had a positive effect on sugar utilization, which was increased from 50.36% to 73.52%, leading to the maximal ethanol concentration and theoretical ethanol yield. Acetic acid and glycerol were formed during ethanol fermentation as observed in this study; however, they were detected at low amount, which were comparable to other studies.^{68–70} In this study, SSJ supplemented only with $(\text{NH}_4)_2\text{SO}_4$ was directly

Table 6 – Comparison of ethanol production by *S. cerevisiae* KKU-VN8 and other yeast strains reported in the literatures.

Strain	S ₀ (g/L)	Sugar source	T (°C)	P (g/L)	SC (%)	T.Y. (%)	Reference
<i>K. marxianus</i> DMKU 3-1042	220	Sugar cane juice	40	67.8	N/A	60.4	Limtong et al. ³
<i>I. orientalis</i> IPE 100	150	Glucose	42	65.5	N/A	85.5	Kwon et al. ¹⁷
<i>P. kudriavzevii</i> DMKU 3-ET15	180	Cassava starch hydrolysate	40	78.6	N/A	85.5	Yuangsaard et al. ¹⁵
<i>S. cerevisiae</i> NRRL Y-2034	200	Sweet sorghum juice	30	70.6	N/A	N/A	Phutela and Kaur ⁶⁷
<i>S. cerevisiae</i> AT-3	180	Glucose	40	68.5	90.0	75.0	Tofighi et al. ⁶⁶
<i>S. cerevisiae</i> SEMF1	185	Sweet sorghum juice	33	86.2	97.3	91.9	Zhao et al. ²⁸
<i>S. cerevisiae</i> DBKKU Y-53	250	Sweet sorghum juice	40	85.01	79.35	77.92	Nuanpeng et al. ³⁰
<i>S. cerevisiae</i> KKU-VN8	238.52	Sweet sorghum juice	40	89.32	73.52	96.32	This study

S₀, initial sugar concentration (g/L); P, ethanol concentration (g/L); SC, Sugar consumption (%); T.Y., theoretical yield of ethanol (%); N/A, not available.

used by *S. cerevisiae* KKU-VN8 to produce ethanol, which differs from other conditions reported in the literature.^{3,15,24–28,30,66}

Conclusions

In this study, five thermotolerant yeast strains, specifically *P. kudriavzevii* KKU-TH33 and KKU-TH43 and *S. cerevisiae* KKU-VN8, KKU-VN20 and KKU-VN27, were successfully isolated. Enhanced stress tolerance levels were observed at high temperatures up to 45 °C and ethanol concentrations up to 13% (v/v). The ethanol production capabilities of these strains were greater than that of the reference strain at elevated temperatures. Maximal ethanol production at 37 °C was achieved by *S. cerevisiae* KKU-VN8, which produced an ethanol concentration of 72.69 g/L, a productivity of 1.59 g/L/h and an ethanol yield of 0.44 g/g using glucose as a substrate. When SSJ only was employed as a raw material, this strain also exhibited the highest ethanol production at 40 °C, with the ethanol concentration, the productivity and the yield of 48.51 g/L, 1.01 g/L/h and 0.43 g/g, respectively. The BBD was used to identify the optimal conditions for ethanol production from SSJ at high temperatures by *S. cerevisiae* KKU-VN8. When the initial yeast cell concentration of 3×10^8 cells/mL was used, the maximal ethanol concentration of 89.32 g/L with the productivity and the theoretical ethanol yield of 2.48 g/L/h and 96.32%, respectively, were achieved under the optimized conditions. Ethanol concentration increased by approximately 45.7% when compared to the unoptimized condition. Our findings provide significant information regarding this candidate yeast for use as an ethanol producer and represent a promising starting point to improve ethanol production processes in the near future. To gain further insights into the molecular mechanisms by which yeast cells adapt to adverse environmental conditions and acquire thermotolerance during ethanol fermentation at high temperatures, the expression of genes involved in specific metabolic processes such as carbohydrate and fatty acid metabolism, the heat

shock response, oxidative stress defense, cellular signaling and protein folding and degradation will also be determined.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bjm.2017.01.006](https://doi.org/10.1016/j.bjm.2017.01.006).

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