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ORIGINAL ARTICLE

Isolation and characterization of thermotolerant yeasts for the production of second-generation bioethanol

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Abstract

The purpose of this study was to isolate, identify, and characterize the thermotolerant yeasts for use in high-temperature ethanol fermentation. Thermotolerant yeasts were isolated and screened from soil samples collected from the Mekong Delta, Vietnam, using the enrichment method. Classification and identification of the selected thermotolerant yeasts were performed using matrix-assisted laser desorption ionization/time-of-fight mass spectrometry (MALDI-TOF/MS) and nucleotide sequencing of the D1/D2 domain of the 26S rDNA and the internal transcribed spacer (ITS) 1 and 2 regions. The ethanol production by the selected thermotolerant yeast was carried out using pineapple waste hydrolysate (PWH) as feedstock. A total of 174 yeast isolates were obtained from 80 soil samples collected from 13 provinces in the Mekong Delta, Vietnam. Using MALDI-TOF/MS and nucleotide sequencing of the D1/D2 domain and the ITS 1 and 2 regions, six different yeast species were identified, including *Meyerozyma caribbica*, *Saccharomyces cerevisiae*, *Candida tropicalis*, *Torulaspora globosa*, *Pichia manshurica*, and *Pichia kudriavzevii*. Among the isolated thermotolerant yeasts, *P. kudriavzevii* CM4.2 displayed great potential for high-temperature ethanol fermentation. The maximum ethanol concentration (36.91 g/L) and volumetric ethanol productivity (4.10 g/L h) produced at 45 °C by *P. kudriavzevii* CM4.2 were achieved using PWH containing 103.08 g/L of total sugars as a feedstock. These findings clearly demonstrate that the newly isolated thermotolerant yeast *P. kudriavzevii* CM4.2 has a high potential for second-generation bioethanol production at high temperature.

Keywords Ethanol production · High-temperature fermentation · *Pichia kudriavzevii* · Pineapple waste · Second-generation bioethanol · Thermotolerant yeasts

Introduction

Ethanol is one of the most popular sources of alternative energy because it can be mixed with petrol to increase the heat of vaporization and octane number. More than 90% of ethanol currently used in the fuel market is produced from sugar- or starch-based materials, such as sugarcane, cassava, and corn (Gombert and van Maris 2015), which may compete with and increase the price of these materials used for food for human needs and animal feed (Farrell et al. 2006). Agricultural wastes provide an abundant renewable resource for second-generation bioethanol production due to their high sugar content and wide availability without competing with the increasing food demand from food crops (Saini et al. 2015; Aditiya et al. 2016; Rastogi and Shrivastava 2017; Robak and Balcerek 2018; Carrillo-Nieves et al. 2019). Pineapple is an economic crop with a worldwide production of approximately 24.79 million metric tons per year. During the production process, approximately 50% of the pineapple is discarded as

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wastes, including pineapple peels, cores, stems, and leaves (Ketnawa et al. 2012). Among these, pineapple peel has been shown to be a promising raw material for ethanol production, since it is highly biodegradable and rich in proteins and carbohydrates (Choonut et al. 2014).

High-temperature ethanol fermentation using thermotolerant yeasts offers several advantages including higher yields of saccharification and fermentation products, a decreased energy requirement for product recovery, and a reduction of cooling costs and contamination (Arora et al. 2015). Several yeast species are currently used industrially or may have potential application in ethanol production, such as *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, *Kluyveromyces marxianus*, and *Candida shehatae*. *S. cerevisiae* is well known as an industrial ethanol producer, with the ability to ferment under both anaerobic and aerobic conditions and accumulate high levels of ethanol (Radecka et al. 2015; Nuanpeng et al. 2016). *P. kudriavzevii* has been isolated from a variety of niches and is known as a typical thermal- and furfural-tolerant yeast (Kurtzman et al. 2011). It has been widely used in the production of many biological products, such as single-cell protein (Rachamontree et al. 2015), biodiesel (Sankh et al. 2013), D-xylonate (Toivari et al. 2013), and phytase (Hellström et al. 2012). Although there are many reports describing ethanol production using thermotolerant yeasts (Yuangsaard et al. 2013; Charoensopharat et al. 2015; Nuanpeng et al. 2016; Chamnipa et al. 2018), only a few studies have considered the isolation of thermotolerant yeasts for high-temperature ethanol fermentation from the Mekong Delta, Vietnam (Techaparin et al. 2017). In this study, thermotolerant yeasts isolated from soil samples collected from 13 provinces in the Mekong Delta, Vietnam, were isolated, identified, and characterized. The ethanol production efficiency of the selected thermotolerant yeasts at high temperature using pineapple waste hydrolysate (PWH) as a raw material was also investigated.

Materials and methods

Raw material and yeast strain

Pineapple wastes (pineapple peels) were collected from the Food Services Center, Khon Kaen University, Thailand. These raw materials were chopped into small pieces, sun dried for 3 days and finally dried in a hot air oven at 80 °C for 24 h. The resulting raw materials were milled and stored at room temperature prior to use. The PWH was prepared using the method as described by Rattanapoltee and Kaewkannetra (2014). Briefly, 0.5% (*v*/*v*) H_2SO_4 solution was added into 200 g/L of dried pineapple waste and pre-treated at 121 °C for 15 min. After hydrolysis, the pellet was filtered and the resulting supernatant (referred to as PWH) was collected and kept at − 20 °C. The chemical compositions of the PWH consisted of 103.08 g/L of total sugars, including 41.18 g/L of glucose, 46.15 g/L of fructose, 4.61 g/L of xylose, and 4.46 g/L of arabinose. Some minerals, such as nitrogen (686 mg/L), phosphorus (274 mg/L), magnesium (126 mg/L), potassium (4344 mg/L), manganese (34 mg/L), and zinc (5 mg/L), and some inhibitors, such as acetic acid (8.54 g/L), formic acid (0.96 g/L), and furfural (0.4 mg/L), were also present in the PWH. The thermotolerant yeast *K. marxianus* DBKKU Y-102 (Charoensopharat et al. 2015) was used as a reference strain in this study.

Isolation and characterization of thermotolerant yeasts

Soil samples from fruit gardens (citrus, jackfruit, rambutan, and mango), agricultural farms (cassava, sweet potato, sugarcane, and pineapple), and sugarcane factories were collected from 13 provinces in the Mekong Delta, Vietnam, including An Giang, Bac Lieu, Ben Tre, Ca Mau, Can Tho, Dong Thap, Hau Giang, Kien Giang, Long An, Soc Trang, Tien Giang, Tra Vinh, and Vinh Long. The thermotolerant yeasts were isolated at 35 °C using the enrichment method (Yuangsaard et al. 2013). Pure cultures of the isolated yeasts were maintained on yeast malt extract (YM) agar at 4 °C.

The thermo-, ethanol, and acetic acid tolerance of the isolated yeasts were determined using the streak plate and drop plate techniques. For the thermotolerance test, yeasts were grown on YM agar plates containing 4% (*v*/*v*) ethanol and were incubated at 37, 40, 43, and 45 °C for 24 h. For the ethanol tolerance test, yeasts were grown on YM agar plates containing 8, 10, 12, and 14% (*v*/*v*) ethanol. For the acetic acid tolerance test, yeasts were grown on YM agar plates containing 4, 6, 8, and 10 g/L acetic acid. Yeast cultures were inoculated onto the agar plates, which were then sealed with plastic wrap and incubated at 35 °C for 24 h. Yeast growth was monitored and photographed.

Screening and selection of thermotolerant ethanol-fermenting yeasts

Yeasts with high potential thermotolerance and ethanol fermentation activity screened for at 37 °C in test tubes containing 10 mL of YM broth (160 g/L D-glucose, pH 5.0) (Nuanpeng et al. 2016). For each isolate, a single yeast colony from a YM agar plate incubated for 18 h was inoculated into a test tube and cultured on a rotary shaker (150 rpm) at 37 °C. After 60 h of fermentation, culture broths were withdrawn and centrifuged, and the ethanol concentration in the clear supernatant was determined via gas chromatography (GC) as described by Nuanpeng et al. (2016). The yeast isolates that produced high levels of ethanol were selected, and their carbon utilization was further analyzed using Biolog MicroStation (Biolog Inc., USA).

Classification and identification of the thermotolerant yeasts

Morphological analysis of all isolates was performed using the standard method described by Kurtzman et al. (2011). All yeast isolates were classified using whole cell matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF/MS; Ultraflex, Bruker Daltonics, USA). The obtained spectra were analyzed to create the dendrogram using BioTyper (Bruker Daltonics) (Tani et al. 2015).

To identify the selected thermotolerant yeasts, nucleotide sequencing of the D1/D2 domain of the 26S rDNA was performed using the specific primers NL-1 (5’-GCATATCAATAAGC GGAGGAAAAG-3′) and NL-4 (5’-GGTCCGTGTTTCA AGACGG-3′) (O’Donnell 1993). Further confirmation of the yeast species was performed by nucleotide sequencing of the internal transcribed spacer (ITS) 1 and 2 regions using the specific primers ITS1 (5’-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5’-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990). The nucleotide sequences were determined using an automated Beckman Coulter sequencer (GenomeLab GeXP, USA) and were compared to the type strains in National Center for Biotechnology Information (NCBI). The nucleotide sequences were aligned using CLUSTAL W, and a phylogenetic tree was constructed using the neighbor-joining method with MEGA version 6.0 with a bootstrap number of 1000 (Tamura et al. 2013).

Ethanol production of the selected thermotolerant yeasts using YM medium

The ethanol fermentation efficiency of the selected thermotolerant yeasts was evaluated in 250-mL flasks using YM medium (pH 5.0) containing 160 g/L glucose. The yeast inocula were grown in YM medium at 35 °C, 150 rpm for 18 h, and were transferred into 250-mL Erlenmeyer flasks containing 100 mL of YM medium to an initial cell concentration of 1×10^7 cells/mL. The flasks were incubated at 37, 40 and 45 °C on a rotary shaker at 100 rpm, with samples taken every 12 h for ethanol and total sugar analyses. The isolate producing the highest ethanol concentration, volumetric ethanol productivity and ethanol yield was selected for further study.

Optimization of ethanol production from PWH by the selected thermotolerant yeast using a statistical experimental design

The optimization experiments were conducted in 250-mL Erlenmeyer flasks containing 100 mL of PWH (pH 5.0). The effect of fermentation factors, including $(NH_4)_2SO_4$ (A), $MnSO_4{\cdot}H_2O$ (B), $MgSO_4{\cdot}7H_2O$ (C), $ZnSO_4{\cdot}7H_2O$ (D), KH_2PO_4 (E), yeast extract (F), pH (G), and initial cell concentration (H) as the independent variables on the ethanol production of the selected thermotolerant yeast was evaluated using a Plackett–Burmam design (PBD). Two levels of each independent variable, i.e., A (0.05 and 3.0 g/L), B (0.02 and 1.5 g/L), C (0.05 and 2.0 g/L), D (0.02 and 1.5 g/L), E (0.05 and 2.0 g/L), F (0 and 6.0 g/L), G (4.0 and 6.0), and H (1×10^6 and 1×10^8 cells/mL) were tested. The significant variables selected based on the PBD were subsequently evaluated and optimized using response surface methodology (RSM) based on the central composite design (CCD). The ethanol concentration (*P*, g/L) was used as the response function in this study.

Analytical methods and data analysis

The viable yeast cell number was determined using a hemacytometer and a methylene blue staining technique (Zoecklien et al. 1995). The total sugar concentrations were measured using the phenol sulfuric acid method (Mecozzi 2005). The ethanol concentration was analyzed by GC (Shimadzu GC-14B, Japan) using polyethylene glycol (PEG-20M) packed column with a flame ionization detector. The sugar compositions (glucose, fructose, xylose, and arabinose) and inhibitors (acetic acid, formic acid, and furfural) of the PWH were analyzed using high-performance liquid chromatography (HPLC) (Shimadzu, Japan) equipped with an Aminex HPX-87H column. Sugars were detected using a refractive index detector, while acids and furfural were detected using the UV-VIS detector at the wavelength of 210 nm. Sulfuric acid (H_2SO_4) (5 mM) was used as a mobile phase at the flow rate of 0.6 mL/min at 40 °C. Minerals in the PWH were analyzed using atomic absorption spectroscopy according to the AOAC standard method. Ethanol yield ($Y_{p/s}$, g/g) was calculated as the actual ethanol produced and was expressed as g ethanol per g sugar utilized. Volumetric ethanol productivity (Q_p, g/L h) was calculated using the following equation: $Q_p = P/t$, where P is the ethanol concentration (g/L) and t is the fermentation time (h) giving the greatest ethanol concentration. The ethanol fermentation efficiency (*Ey*, %) was calculated by the following equation: $Ey = (Yp/s/0.511) \times 100$, where Yp/s is the ethanol yield (g/g), and 0.511 is the theoretical maximum ethanol yield per unit of glucose from glycolytic fermentation (g/g). The results were expressed as the mean ± standard deviation (SD) values from three replicates. Statistical analyses were performed using Statgraphics Centurion XV (Statpoint Technologies, Inc., USA). Analysis of variance (ANOVA) was used to evaluate the differences among the treatments using Duncan’s multiple range tests (DMRTs).

Results

Isolation and characterization of thermotolerant yeasts

A total number of 174 yeast isolates were obtained from 80 soil samples collected in the Mekong Delta, Vietnam.

Morphological analysis revealed that most colonies had a smooth surface, while some colonies displayed a rough surface with a white or creamy white color. The margins of the colonies were primarily entire, serrated, or lobate. The cell shapes of the yeast isolates varied, e.g., spherical, ovoid, elongated ellipse, and cylinder with the lengths of 3–11 μm and the widths of 2–6 μm (data not shown). All isolates were tested for their tolerance to high temperature stress, and the results showed that all the isolates could grow at 37 °C. At 40 and 43 °C, 157 (~ 90%) and 135 isolates (~ 78%) could grow, respectively, while only 20 isolates (~ 12%) were able to grow at 45 °C. With respect to ethanol stress, 149 isolates (~ 86%) grew well in YM medium containing 10% (*v*/*v*) ethanol. However, only 61 (~ 35%) and 30 isolates (~ 17%) could grow in YM medium containing 12 and 14% (*v*/*v*) ethanol, respectively. Based on the growth performance of the isolated yeasts at a relatively high temperature of 40 °C and an ethanol concentration of 12% (*v*/*v*) (Supplementary Fig. 1), 57 isolates of yeast (~ 33%) were chosen for further experiments.

Screening and selection of thermotolerant ethanol fermentative yeasts

Fifty-seven thermotolerant yeast isolates were evaluated for their ethanol fermentation capability using YM medium containing 160 g/L of glucose as the sole carbon source. All of the selected thermotolerant yeasts were able to produce ethanol at 37 °C, with the ethanol concentrations varying from 23.16 to 48.71 g/L after 60 h of fermentation. Only 8 isolates (designated as BL5.1, HG1.1, CM4.2, HG1.2, DT5.3, CT5.3, TG5.3, and ST1.1) produced ethanol at the concentrations higher than 40 g/L, which were comparable to those of the reference strain, *K. marxianus* DBKKU Y-102 (44.40 g/L) (data not shown). By using carbon assimilation analysis, it was shown that the isolates HG1.1 and HG1.2 could assimilate several types of carbon sources, including glucose, sucrose, galactose, trehalose, maltose, raffinose, melibiose, and ethanol, while the other six isolates could utilize only glucose, ethanol, glycerol, and N-acetyl-D-glucosamine (Supplementary Table 1).

The growth characteristics of the eight thermotolerant yeast isolates were determined at various temperatures (Fig. 1a). Six isolates, including BL5.1, CM4.2, CT5.3, DT5.3, ST1.1, and TG5.3, could grow at 37, 40, 43, and 45 °C, although their growth was lower than that of the reference strain at 43 and 45 °C. HG1.1 and HG1.2 were able to grow at 37 and 40 °C, but their growth was almost inhibited at 43 and 45 °C. With respect to ethanol stress, all of the assayed yeasts could grow in medium containing 8, 10, 12, and 14% (*v*/*v*) ethanol. In contrast, the reference strain was able to grow in the medium containing 8, 10, and 12% (*v*/*v*) ethanol, but its growth was completely inhibited in the medium containing 14% (*v*/*v*) ethanol (Fig. 1b). With respect to acetic acid stress, all isolates could grow in the medium containing 4 g/L acetic acid. However, only six isolates, including BL5.1, CM4.2, CT5.3, DT5.3, ST1.1, and TG5.3, could grow in the medium containing 6 and 8 g/L acetic acid. Interestingly, only one isolate, CM4.2, could grow in the medium containing 10 g/L acetic acid. The isolates HG1.1 and HG1.2 and the reference strain could not grow in the medium containing acetic acid at the concentrations higher than 4 g/L (Fig. 1c).

Classification and identification of the thermotolerant yeasts

Based on the whole-cell MALDI-TOF/MS identification, all 174 thermotolerant yeast isolates were classified into six groups, including *M. caribbica*, *S. cerevisiae*, *C. tropicalis*, *T. globosa*, *P. manshurica*, and *P. kudriavzevii* (Supplementary Fig. 2). To confirm this result, further identification of the yeast species by sequencing the D1/D2 domain of the 26S rDNA and the ITS1 and ITS2 regions was conducted. Only 35 isolates, including eight representative isolates from the 6 groups and 27 isolates exhibiting a high tolerance to heat (40 °C), ethanol (14% *v*/*v*) and acetic acid (4 g/L) were used for this confirmation. Phylogenetic analysis of the DNA sequences revealed that the 35 selected isolates clustered into six groups, including *M. caribbica*, *S. cerevisiae*, *C. tropicalis*, *T. globosa*, *P. manshurica*, and *P. kudriavzevii*, which was the same as those observed by the whole-cell MALDI-TOF/MS (Supplementary Fig. 3). Notably, eight isolates that produced a high concentration of ethanol (more than 40 g/L) at high temperatures were identified as two yeast species, *P. kudriavzevii* (BL5.1, CM4.2, DT5.3, CT5.3, ST1.1, and TG5.3) and *S. cerevisiae* (HG1.1 and HG1.2).

Ethanol production by the selected thermotolerant yeasts using YM medium

The production of ethanol at high temperatures by *P. kudriavzevii* BL5.1, CM4.2, DT5.3, CT5.3, ST1.1, and TG5.3 and *S. cerevisiae* HG1.1 and HG1.2 using YM medium containing 160 g/L glucose was evaluated, and the results are summarized in Table 1. *P. kudriavzevii* CM4.2 produced the highest ethanol concentration, volumetric ethanol productivity, and ethanol yield with a relatively high fermentation efficiency at 37, 40, and 45 °C compared to the other isolates and the reference strain. The maximum ethanol concentrations produced by *P. kudriavzevii* CM4.2 at 37, 40, and 45 °C were 72.47, 71.98, and 47.76 g/L, which were approximately 26, 37, and 27% greater than that of the reference strain, respectively. Two *S. cerevisiae* isolates (HG1.1 and HG1.2) produced relatively high levels of ethanol at 37 and 40 °C, but their fermentation activity decreased remarkably at 45 °C. The ethanol concentrations and productivities produced by these two *S. cerevisiae* strains were approximately 55–56% and 77–

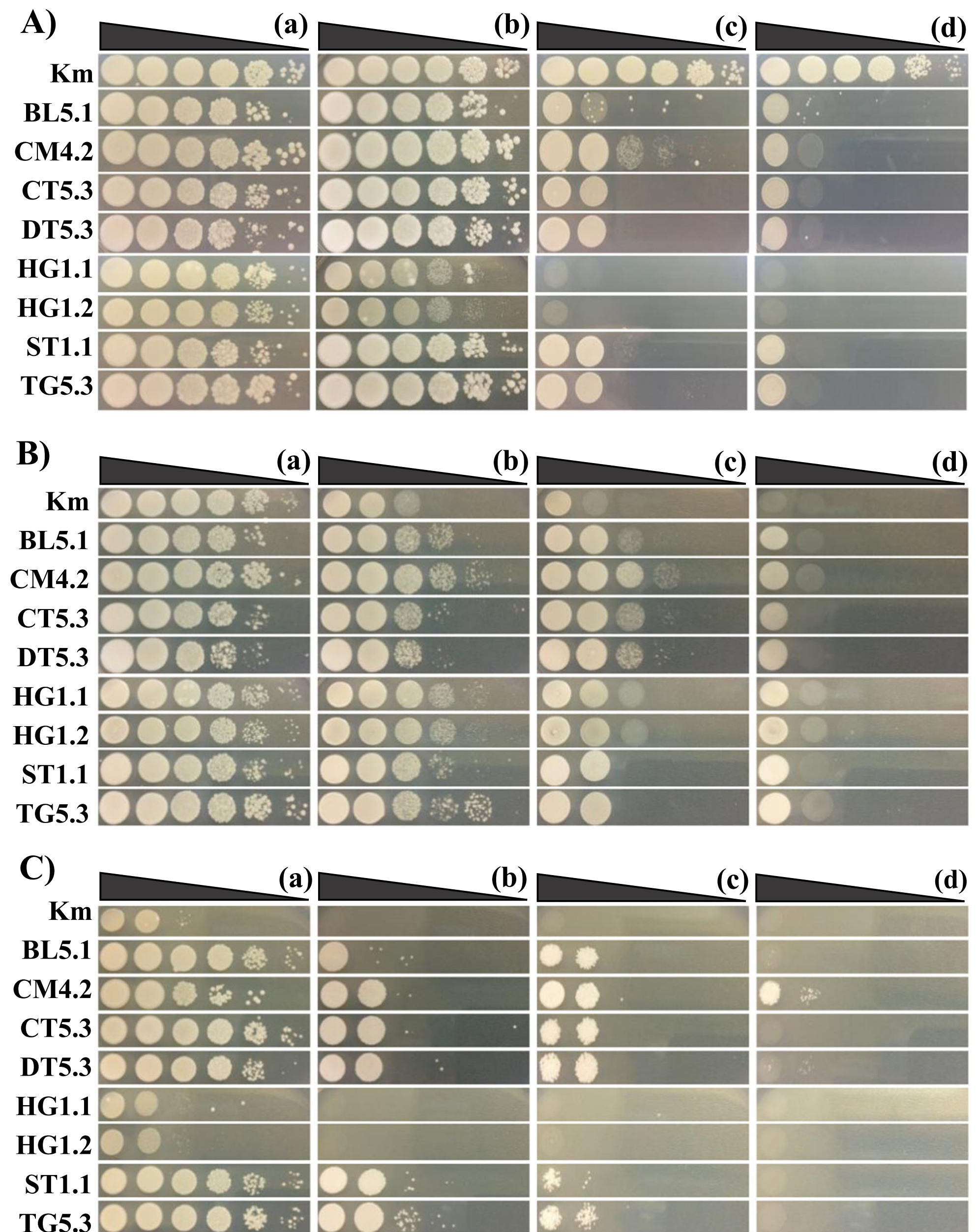


Fig. 1 Growth of *K. marxianus* DBKKU Y-102 (Km), *P. kudriavzevii* (BL5.1, CM4.2, CT5.3, DT5.3, ST1.1, and TG5.3), and *S. cerevisiae* (HG1.1 and 1.2) on YM agar medium supplemented with: (**A**) ethanol at a concentration of 4%% (*v*/*v*) and incubated at 37 °C (a), 40 °C (b), 43 °C (c), and 45 °C (d); (**B**) ethanol at a concentration of 8% (a), 10% (b), 12% (c), and 14% (*v*/*v*) (d) and incubated at 35 °C; and (**C**) acetic acid at a concentration of 4 g/L (a), 6 g/L (b), 8 g/L (c), and 10 g/L (*v*/*v*) (d) and incubated at 35 °C for 24 h

78% lower than that of the reference strain at 45 °C, respectively. Based on these findings, *P. kudriavzevii* CM4.2 was chosen for further study.

Optimization of ethanol production from PWH by *P. kudriavzevii* CM4.2

The optimum condition for ethanol production by *P. kudriavzevii* CM4.2 using PWH was investigated using a statistical experimental design. The PBD was used to evaluate the significance of each independent variable for ethanol production at a high temperature (45 °C). The experimental design matrices and the response variable from 12 experimental runs are summarized in Supplementary Table 2. The highest ethanol concentration (31.40 g/L) and volumetric ethanol productivity (2.62 g/L h) was achieved from experimental run no. 8 (3.0 g/L $(NH_4)_2SO_4$, 0.02 g/L $MnSO_4 \cdot H_2O$, 0.05 g/L $MgSO_4 \cdot 7H_2O$, 0.02 g/L $ZnSO_4 \cdot 7H_2O$, 2.0 g/L KH_2PO_4, pH 6.0 and 1×10^8 cells/mL), while the lowest concentration (0.97 g/L) and volumetric ethanol productivity (0.08 g/L h) of ethanol was achieved from experimental run no.7 (3.0 g/L $(NH_4)_2SO_4$, 1.50 g/L $MnSO_4 \cdot H_2O$, 0.05 g/L $MgSO_4 \cdot 7H_2O$, 1.50 g/L $ZnSO_4 \cdot 7H_2O$, 2.0 g/L KH_2PO_4, 6 g/L yeast extract, pH 4.0 and 1×10^6 cells/mL). The reliability of the established model for PBD was tested using ANOVA. As shown in Table 2, the *p* value of the established model was less than 0.05 (0.0009), indicating that the model was highly significant and could be used to identify the significant variables affecting the ethanol production. According to the *p* value from ANOVA, four

Table 1 Ethanol production at 37, 40, and 45 °C by *K. marxianus* DBKKU Y-102 (Km), *P. kudriavzevii* (BL5.1, CM4.2, CT5.3, DT5.3, ST1.1, and TG5.3), and *S. cerevisiae* (HG1.1 and 1.2) using YM medium containing 160 g/L glucose

Yeast Isolate	Parameters (mean ± SD)			
	P (g/L)	Q_p (g/L h)	$Y_{p/s}$ (g/g)	E_y (%)
37 °C				
Km	53.54 ± 1.56d	1.12 ± 0.04d	0.35 ± 0.01c	69.85 ± 0.43d
BL5.1	70.67 ± 1.78ab	1.47 ± 0.04a	0.47 ± 0.01ab	92.20 ± 1.84ab
CM4.2	72.47 ± 0.13a	1.51 ± 0.01a	0.48 ± 0.00a	94.56 ± 0.18a
CT5.3	71.04 ± 0.86ab	1.48 ± 0.02a	0.48 ± 0.01a	92.68 ± 1.11ab
DT5.3	68.17 ± 0.54c	1.14 ± 0.01cd	0.46 ± 0.01b	88.94 ± 0.70c
HG1.1	71.42 ± 0.33ab	1.49 ± 0.01a	0.48 ± 0.01a	93.18 ± 0.42ab
HG1.2	71.89 ± 0.78ab	1.20 ± 0.02b	0.48 ± 0.01a	93.79 ± 1.02ab
ST1.1	72.47 ± 0.22a	1.21 ± 0.01b	0.48 ± 0.00a	94.54 ± 0.28a
TG5.3	69.78 ± 0.41bc	1.17 ± 0.01bc	0.47 ± 0.01ab	91.04 ± 0.54bc
40 °C				
Km	44.89 ± 0.64e	0.94 ± 0.01f	0.30 ± 0.00e	64.57 ± 0.83e
BL5.1	68.09 ± 1.71bc	1.42 ± 0.03bc	0.46 ± 0.02bc	88.83 ± 1.52bc
CM4.2	71.98 ± 0.90a	1.50 ± 0.01a	0.48 ± 0.01a	93.77 ± 0.99a
CT5.3	70.13 ± 0.52ab	1.46 ± 0.01ab	0.47 ± 0.01ab	91.49 ± 0.67ab
DT5.3	67.43 ± 0.86bc	1.41 ± 0.02bc	0.45 ± 0.01bc	87.98 ± 1.12bc
HG1.1	67.20 ± 1.74bc	1.40 ± 0.03bc	0.45 ± 0.02bc	87.68 ± 1.26bc
HG1.2	60.90 ± 1.23d	1.02 ± 0.02e	0.41 ± 0.02d	79.45 ± 1.17d
ST1.1	66.33 ± 0.05c	1.11 ± 0.01d	0.44 ± 0.01c	86.53 ± 0.07c
TG5.3	66.94 ± 0.01bc	1.37 ± 0.02c	0.45 ± 0.01bc	87.33 ± 0.01bc
45 °C				
Km	34.78 ± 0.57c	1.45 ± 0.02a	0.32 ± 0.01b	61.87 ± 0.74c
BL5.1	32.22 ± 1.86c	0.54 ± 0.03d	0.32 ± 0.02b	63.05 ± 0.52c
CM4.2	47.76 ± 1.47a	1.00 ± 0.02b	0.46 ± 0.01a	89.05 ± 1.21a
CT5.3	40.90 ± 0.76b	0.85 ± 0.01bc	0.41 ± 0.01a	80.03 ± 0.99b
DT5.3	31.24 ± 1.96c	0.87 ± 0.03bc	0.31 ± 0.02b	61.14 ± 0.32c
HG1.1	15.52 ± 1.81d	0.33 ± 0.02e	0.16 ± 0.02c	30.38 ± 0.21d
HG1.2	15.21 ± 1.28d	0.32 ± 0.03e	0.15 ± 0.01c	29.84 ± 0.16d
ST1.1	43.32 ± 0.55ab	0.73 ± 0.01c	0.44 ± 0.01a	84.77 ± 0.72ab
TG5.3	30.16 ± 1.05c	0.50 ± 0.01d	0.30 ± 0.02b	59.01 ± 1.25c

The mean values with different letters in the same column are significantly different at $p < 0.05$ based on DMRT analysis

P ethanol concentration (g/L), Q_p volumetric ethanol productivity (g/L h), $Y_{p/s}$ ethanol yield (g/g), E_y fermentation efficiency (%)

independent variables, including $(NH_4)_2SO_4$ (A), $MnSO_4 \cdot H_2O$ (B), pH (G), and initial cell concentration (H) were highly significant parameters affecting the ethanol production from PWH using *P. kudriavzevii* CM4.2. The significances of all of the independent variables were further confirmed using a Pareto chart analysis. Based on the t value limit of the experiment, four independent variables (A, B, G, and H) were considered significant parameters, similar to the ANOVA results. The variables A, G, and H showed a positive effect on ethanol production, while variable B exerted a negative effect (Fig. 2). Based on these findings, these four variables were chosen for the optimization experiments.

The optimum condition for ethanol production from PWH using *P. kudriavzevii* CM4.2 at 45 °C was determined using RSM based on the CCD. The experimental design matrices and the observed values for the ethanol concentrations and volumetric ethanol productivities are summarized in Supplementary Table 3. The observed ethanol concentrations varied from 9.56 to 32.93 g/L, while the volumetric ethanol productivities varied from 0.80 to 2.74 g/L h. The statistical analysis showed that the established model was highly significant since the p value was less than 0.05 (Table 3). Based on the ANOVA, the initial cell concentration (A), pH (B),

Table 2 Analysis of variance (ANOVA) for the Plackett–Burman design

Source	Sum of squares	*df*	Mean square	*F* value	*p* value Prob > *F*	Note
Model	1652.52	8	206.56	138.27	0.0009	Significant
A-$(NH_4)_2SO_4$	26.85	1	26.85	17.97	0.0240	Significant
B-$MnSO_4·H_2O$	37.00	1	37.00	24.76	0.0156	Significant
C-$MgSO_4·7H_2O$	2.30	1	2.30	1.54	0.3031	
D-$ZnSO_4·7H_2O$	5.45	1	5.45	3.65	0.1520	
E-KH_2PO_4	0.23	1	0.23	0.16	0.7196	
F-yeast extract	0.90	1	0.90	0.60	0.4938	
G-pH	43.66	1	43.66	29.23	0.0124	Significant
H-cell	1536.12	1	1536.12	28.22	< 0.0001	Significant
Residual	4.48	3	1.49			
Cor total	1657.00	11				
Std. dev.	1.22		R-squared		0.9973	
C.V. (%)	9.20		Adj R-squared		0.9901	

and their interaction (AB) significantly affected the ethanol production by *P. kudriavzevii* CM4.2 at 45 °C.

The four-factor quadratic polynomial equation derived from the quadratic polynomial regression model used to predict the final ethanol concentration (*P*, g/L) was as follows:

$$P\ (\mathrm{g/L}) = 31.87 + 5.24A + 3.66B + 0.016C - 0.18D - 1.47AB + 0.085AC + 0.16AD - 0.52BC + 0.17BD + 0.053CD - 3.10A^2 - 3.20B^2 - 0.42C^2 - 0.38D^2$$

The 3D response surface and contour plots revealed that the most fit model was achieved when the concentrations of $(NH_4)_2SO_4$ (C) and $MnSO_4·H_2O$ (D) were fixed at 1.55 and 0.51 g/L, respectively (Fig. 3). Two parameters, the cell concentration and pH, strongly affected the ethanol production by *P. kudriavzevii* CM4.2. The maximum ethanol concentration (32.93 g/L) and volumetric ethanol productivity (2.74 g/L h) was achieved at the cell concentration of 1.5×10^8 cells/mL and the pH of 5.5.

To verify the optimum values predicted using RSM based on the CCD, repeated experiments were performed to assess ethanol production at 45 °C by *P. kudriavzevii* CM4.2 using the initial cell concentration of 2.2×10^8 cells/mL, the pH of 5.8, and 1.08 and 0.63 g/L of $(NH_4)_2SO_4$ and $MnSO_4·H_2O$, respectively. As shown in Fig. 4, the maximum ethanol concentration of 36.91 g/L was achieved within 9 h of fermentation. The obtained ethanol yield of 0.49 g/g corresponded to the ethanol fermentation efficiency of 95.92%. The observed

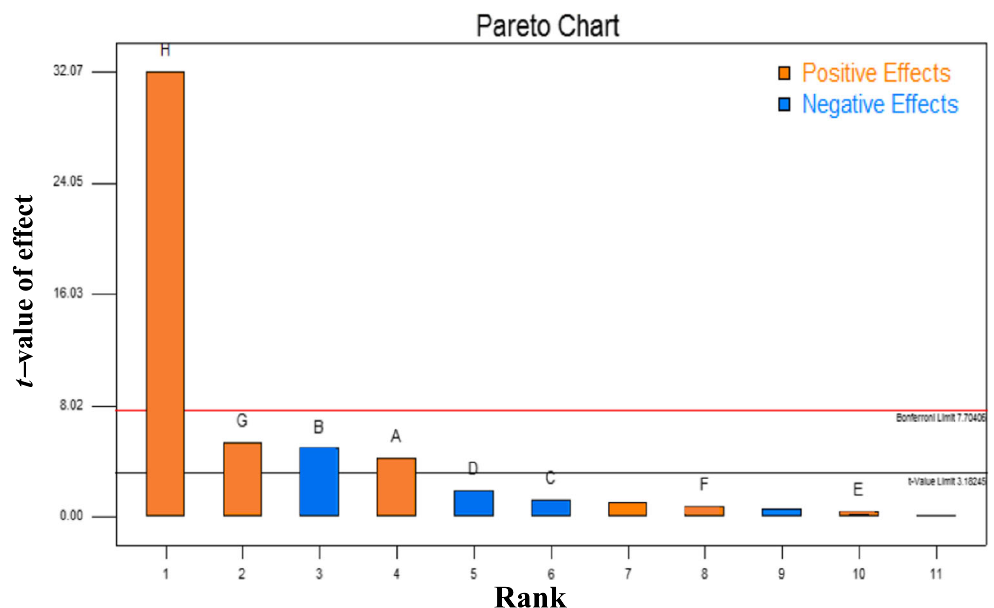


Fig. 2 Pareto chart of standardized effects for a Plackett–Burman design of $(NH_4)_2SO_4$ (A), $MnSO_4·H_2O$ (B), $MgSO_4·7H_2O$ (C), $ZnSO_4·7H_2O$ (D), KH_2PO_4 (E), yeast extract (F), pH (G) and cell concentration (H)

Table 3 Analysis of variance (ANOVA) and the regression analysis results of the CCD on ethanol production using *P. kudriavzevii* CM4.2 at 45 °C

Source	Sum of squares	*df*	Mean square	*F* value	*p* value Prob > *F*	Note
Model	1504.52	14	107.47	38.45	< 0.0001	Significant
A	658.35	1	658.35	235.57	< 0.0001	
B	321.20	1	321.20	114.93	< 0.0001	
C	6.017E−03	1	6.017E−03	2.153E−03	0.9636	
D	0.78	1	0.78	0.28	0.6039	
AB	34.40	1	34.40	12.31	0.0032	
AC	0.12	1	0.12	0.041	0.8416	
AD	0.41	1	0.41	0.15	0.7072	
BC	4.39	1	4.39	1.57	0.2293	
BD	0.44	1	0.44	0.16	0.6964	
CD	0.044	1	0.044	0.016	0.9017	
A^2	264.44	1	264.44	94.62	< 0.0001	
B^2	280.43	1	280.43	100.34	< 0.0001	
C^2	4.95	1	4.95	1.77	0.2029	
D^2	4.01	1	4.01	1.44	0.2494	
Residual	41.92	15	2.79			
Lack of fit	37.48	10	3.75	4.22	0.0628	Not significant
Pure error	4.44	5	0.89			
Cor total	1546.44	29				
Std. dev.	1.67		R-squared		0.9729	
C.V. (%)	6.39		Adj R-squared		0.9476	

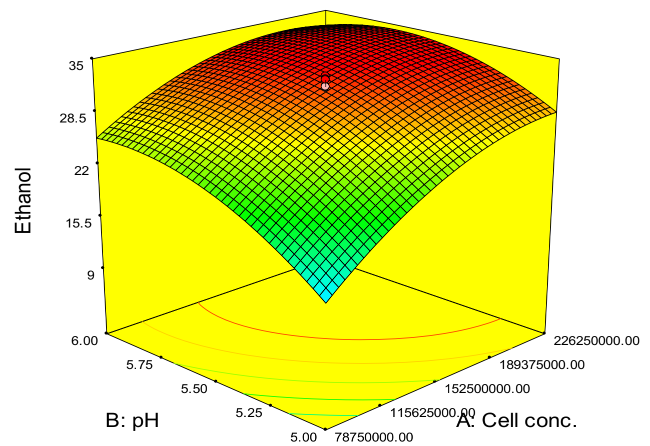
ethanol concentration achieved in this experiment was relatively close to the predicted values, suggesting that the established model was reliable.

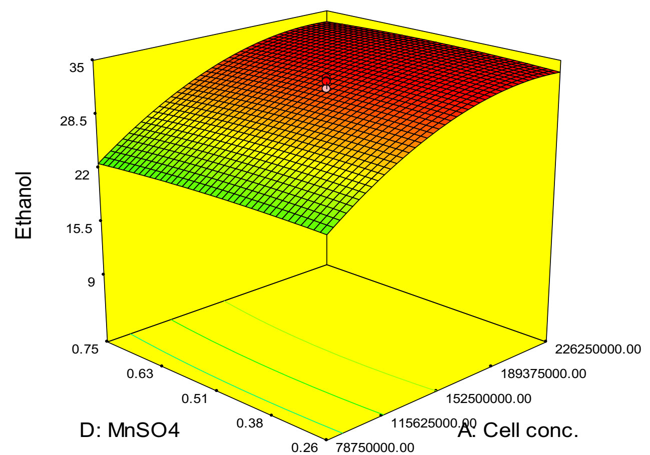
Discussion

The enrichment culture technique has been widely used to isolate useful thermotolerant yeasts for high-temperature ethanol fermentation (Limtong et al. 2007; Yuangsaard et al. 2013; Keo-oudone et al. 2016; Talukder et al. 2016; Choi et al. 2017; Chamnipa et al. 2018). Using this technique, diverse thermotolerant yeasts were successfully isolated from soil samples collected in the Mekong Delta, Vietnam. Several studies have reported the isolation of thermotolerant yeasts from soil samples and obtained several high potential thermotolerant yeasts for high-temperature ethanol fermentation, e.g., *P. kudriavzevii* PBB511-1 (Kaewkrajay et al. 2014), *S. cerevisiae* DBKKUY-53 (Nuanpeng et al. 2016), and *P. kudriavzevii* RZ8-1 (Chamnipa et al. 2018). Although several thermotolerant yeasts have been identified from other sources, e.g., natural fermented products (Talukder et al. 2016), fruits (Keo-oudone et al. 2016), and nuruk (a traditional Korean fermentation starter) (Choi et al. 2017), the present study demonstrated that soil is superior to other sources for isolating thermotolerant yeasts for ethanol production at high temperatures.

The morphological and physiological appearances of the thermotolerant yeasts isolated in the current study were varied depending on the yeast species. However, their characteristics were in good agreement with those described by Kurtzman et al. (2011) and other researchers (Talukder et al. 2016; Techaparin et al. 2017; Chamnipa et al. 2018). The whole-cell MALDI-TOF/MS was used to identify the thermotolerant yeasts (Supplementary Fig. 2). Due to its high sensitivity, cost effectiveness, and rapid processing, this technique has emerged as having a high potential for a number of purposes, such as microbial classification and identification, strain typing, the detection of water- and food-borne pathogens, and the detection of blood and urinary tract pathogens. By either comparing the peptide mass fingerprint (PMF) of an unknown organism with the database PMFs, or by matching the masses of biomarkers of an unknown organism with the proteome database, microbial cells can be identified (Singhal et al. 2015; Tani et al. 2015). In this study, the D1/D2 domain and the ITS regions were also analyzed for microbial identification confirmation at the species levels (Supplementary Fig. 3). Closely related microorganisms, especially those with identical D1/D2 domain DNA sequences, can be differentiated using the ITS1 and ITS2 regions (Chen et al. 2001). Therefore, the classification and identification of yeasts should utilize both sequences.

It should be noted that the thermotolerant yeasts *P. kudriavzevii* and *C. tropicalis* were the most abundant yeast species identified in this study, which have been





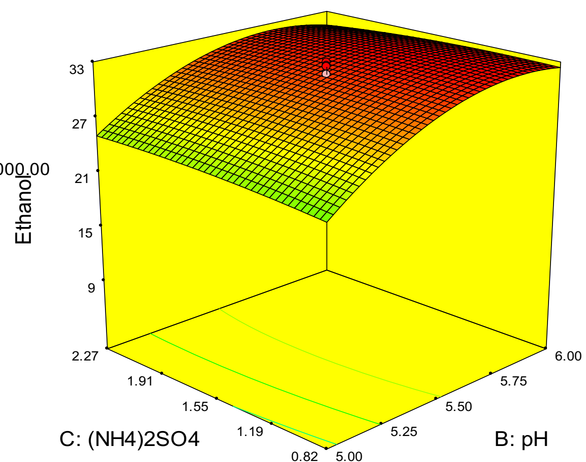


Fig. 3 3D response surface plots showing the effect of cell concentration (A), pH (B), $(NH_4)_2SO_4$ (C), and $MnSO_4 \cdot H_2O$ (D) on the ethanol concentration obtained from PWH using *P. kudriavzevii* CM4.2

isolated from various sources, such as soil (Yuangsaard et al. 2013; Chamnipa et al. 2018), herbivore feces (Lorliam et al. 2013), fermented cocoa (Hamdouche et al. 2015), and naturally fermented products (Talukder et al. 2016). *P. kudriavzevii* has been reported to be a thermotolerant yeast with a high potential for ethanol production at high temperatures using cassava starch hydrolysate (Kaewkrajay et al. 2014) and sugarcane bagasse hydrolysate (Chamnipa et al. 2018). *S. cerevisiae* and *T. globosa* are recognized as thermotolerant yeasts with a high potential for high-temperature ethanol production that can be isolated from soil (Nuanpeng et al. 2016;

Techaparin et al. 2017; Tolieng et al. 2018), sugarcane juices, sediments from sugar process, sugar-rich materials, and fruits (Phong et al. 2016). *P. manshurica* (*P. galeiformis*) has been reported to be a common spoilage yeast species in wines (Saez et al. 2011), although it displayed great potential for acid and ester production in vinegar (Zhang et al. 2017) as well as ethanol production from glucose (Tolieng et al. 2018). *M. caribbica* is recognized as a nontoxic yeast that has been used to produce the alcoholic beverage tequila in Mexico (Saucedo-Luna et al. 2011).

Among the isolated thermotolerant yeasts, *P. kudriavzevii* CM4.2 displayed great potential for ethanol production at high temperatures, growing at a relatively high temperature of 45 °C, an ethanol concentration of 14% (*v*/*v*) and an acetic acid concentration of 10 g/L (Fig. 1). As shown in Table 1, the maximum ethanol concentrations produced by this strain using 160 g/L of glucose were 72.47, 71.98, and 47.76 g/L at 37, 40, and 45 °C, respectively, which were higher than those reported by Kaewkrajay et al. (2014) and Chamnipa et al. (2018). When PWH containing 103.08 g/L of total sugars was used as a raw material for ethanol production by *P. kudriavzevii* CM4.2 at 45 °C, 36.91 g/L of ethanol was achieved under the optimum fermentation condition (Fig. 4).

Several factors influence the growth and ethanol production by yeast, such as the carbon and nitrogen source, pH, incubation temperature, initial yeast cell concentration, and trace elements (Kaewkrajay et al. 2014; Nuanpeng et al. 2016; Techaparin et al. 2017; Chamnipa et al. 2018). To screen the significant factors affecting *P. kudriavzevii* CM4.2 growth and ethanol production at 45 °C using PWH, PBD was employed in this study. This experimental design is recognized as an efficient screening method to identify the important factors using as few experimental runs as possible compared with so-called one factor at a time experiments (Khuri and Mukhopadhyay 2010). As shown in the current study, four independent variables, including $(NH_4)_2SO_4$, $MnSO_4{\cdot}H_2O$, pH and initial cell concentration were identified as the major factors influencing the *P. kudriavzevii* CM4.2 growth and ethanol production from PWH (Table 2). Yeast cell concentration is one of the important fermentation parameters influencing the sugar utilization rate and fermentation efficiency. Previous studies demonstrated that higher initial cell concentrations enhanced the rate and fermentation efficiency for ethanol production from alkali-treated cotton stalks using *P. kudriavzevii* HOP-1 (Kaur et al. 2012), Jerusalem artichoke tubers using *K. marxianus* DBKKU Y-102 (Charoensopharat et al. 2015), and sweet sorghum juice using *S. cerevisiae* KKU-VN8 (Techaparin et al. 2017). The pH value is known as an important factor that directly affects yeast growth and ethanol production. The optimum pH for yeast growth and ethanol production depends on several factors, such as the growth conditions, the yeast species and the raw materials, and in general the optimum pH ranges from 4.0–6.0. In this study, the optimum pH for ethanol production from PWH by *P. kudriavzevii* CM4.2 was 5.8, which was in good agreement with those values reported by Nuanpeng et al. (2016) and Techaparin et al. (2017) using sweet sorghum juice as a feedstock.

Yeast growth and ethanol production also depend on the nitrogen source because it is essential for the synthesis of structural and functional proteins involved in yeast growth and metabolism. Among the various nitrogen sources, inorganic nitrogen, such as urea and $(NH_4)_2SO_4$ have been shown to be suitable for ethanol production (Nuanpeng et al. 2016). In this study, supplementation of $(NH_4)_2SO_4$ enhanced ethanol production from PWH by *P. kudriavzevii* CM4.2. This result was in good agreement with those reported by Limtong et al. (2007) using sugarcane juice and Techaparin et al. (2017) using sweet sorghum juice as a feedstock. Trace elements play important roles in yeast growth and ethanol production by serving as a regulator of many important

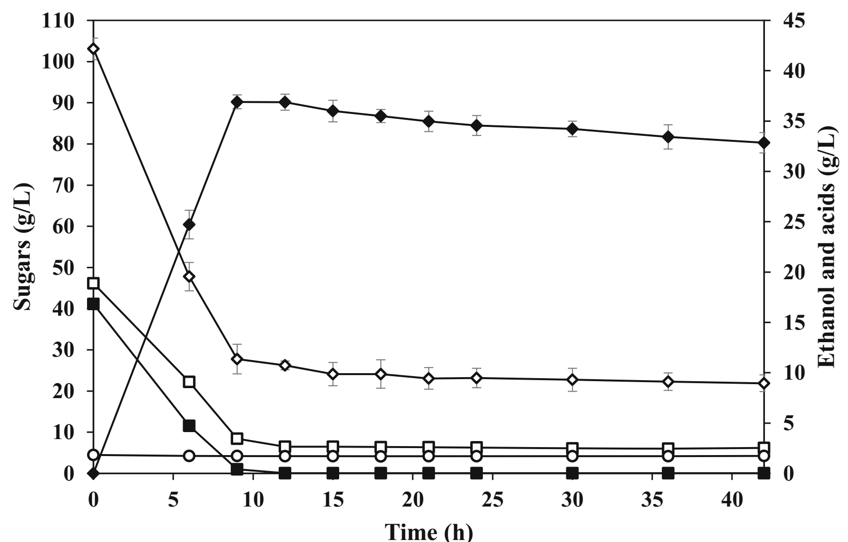


Fig. 4 Time course of ethanol production from PWH at 45 °C using *P. kudriavzevii* CM4.2 under the optimum conditions. Symbols: (filled diamonds) ethanol; (empty diamonds) sugars; (filled squares) glucose; (empty squares) fructose and xylose; and (circles) arabinose

enzymes in microbial cells. Several trace elements have been used to promote the production of ethanol from microbial cells, such as zinc (Zn^{2+}), magnesium (Mg^{2+}), and manganese (Mn^{2+}) (Faga et al. 2010; Deesuth et al. 2012). It should be noted that in this study a number of assayed factors, including yeast extract, $MgSO_4{\cdot}7H_2O$, $ZnSO_4{\cdot}7H_2O$, and KH_2PO_4, had no significant effect on the ethanol production by *P. kudriavzevii* CM4.2, similar with those reported by Rani et al. (2010) and Izmirlioglu and Demirci 2015). One possible reason is that the PWH contains a sufficient amount of organic nitrogen source and trace elements, particularly magnesium, zinc, and potassium. With respect to magnesium, it can decrease the plasma membrane permeability and protect the yeast cells from heat and ethanol stress during ethanol fermentation (Birch and Walker 2000), but it did not promote ethanol production at 45 °C by *P. kudriavzevii* CM4.2. This finding was in good agreement with a report by Techaparin et al. (2017), who used sweet sorghum juice as a feedstock. In addition, Limtong et al. (2007) and Yuangsaard et al. (2013) also observed that $MgSO_4{\cdot}7H_2O$ and KH_2PO_4 did not improve the ethanol production efficiency of *K. marxianus* DMKU 3-1042 using sugarcane juice and of *P. kudriavzevii* DMKU 3-ET15 using cassava starch hydrolysate as a feedstock, respectively.

Conclusions

Diverse yeast species, including *M. caribbica*, *S. cerevisiae*, *C. tropicalis*, *T. globosa*, *P. manshurica*, and *P. kudriavzevii* were isolated from soil samples in the Mekong Delta, Vietnam. Among these isolated yeasts, a newly identified thermotolerant yeast strain, *P. kudriavzevii* CM4.2, displayed great potential for high-temperature ethanol fermentation. This strain could tolerate both high temperatures and ethanol concentrations. Furthermore, it was also highly resistant to high concentrations of acetic acid, making this thermotolerant yeast a very useful organism for ethanol production at high temperatures using lignocellulosic hydrolysate as a feedstock. The maximum ethanol concentration (36.91 g/L) produced at 45 °C by *P. kudriavzevii* CM4.2 using PWH as substrate was achieved in this study. Thus, pineapple waste has a high potential to be used as feedstock for ethanol production at high temperature.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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