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Ethanol production from cassava using a newly isolated thermotolerant yeast strain

Chutima Kaewkrajay[a,*], **Tida Dethoup**[b], **Savitree Limtong**[c]

[a] Faculty of Science and Technology, Phranakhon Si Ayutthaya Rajabhat University, Ayutthaya 13000 Thailand

[b] Faculty of Agriculture, Kasetsart University, Bangkok 10900 Thailand

[c] Faculty of Science, Kasetsart University, Bangkok 10900 Thailand

[*]Corresponding author, e-mail: kchutima@aru.ac.th, pretty_yeast@yahoo.com

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ABSTRACT: Efficient ethanol production using thermotolerant yeast strains was carried out with a newly isolated yeast strain called 267. Strains were isolated with an enriched technique consisting of a blackstrap molasses medium supplemented with 40 ml/l of ethanol at 25–28 °C. The results revealed that 33 strains produced ethanol at 45 °C in the cassava starch hydrolysate medium, pH 4.5, which was composed of 180 g/l reducing sugar and 0.5 g/l $(NH_4)_2SO_4$, with a shaking speed of 120 rpm. The highest ethanol concentrations (26.2, 26.2, 23.6, and 22.5 g/l) were found from *Pichia kudriavzevii* strains PBB511-1, TM512-2, CPY514-1, and TG514-2, respectively. The yeast strain PBB511-1, which produced the highest ethanol yield, was selected for ethanol production in the shaking flask at 45 °C. Ethanol production reached the highest level after 36 h in a medium composed of 180 g/l reducing sugar, 0.5 g/l $(NH_4)_2SO_4$, 0.5 g/l KH_2PO_4, 0.5 g/l $MgSO_4 \cdot 7\,H_2O$, and 1 g/l yeast extract. It produced 37 g/l ethanol, with a productivity of 1.03 (g/l)/h and a yield of 40% of the theoretical yield. The ethanol production by batch fermentation at 45 °C was performed in a 7-l jar fermenter with an agitation speed of 300 rpm and an aeration rate of 0.2 vvm throughout the fermentation. The results implied that the maximum ethanol concentration was 42.4 g/l after 48 h, at a rate of 0.88 (g/l)/h and a yield of 46% of the theoretical yield.

KEYWORDS: *Pichia kudriavzevii*, high temperature, isolation, identification

INTRODUCTION

Due to the current crisis of increasing petroleum fuel prices as well as problems concerning global warming, several countries are seeking alternative energy sources such as ethanol[1–4]. Ethanol is a pure form of energy that has minimal impact to the environment.

For ethanol production in Thailand, a number of raw materials are used such as molasses and cassava[5,6]. Cassava (*Manihot esculenta*), in particular, is considered a main industrial crop. In addition, cassava can grow well in soil with poor fertility. Apart from that, cassava can endure drought conditions, and can also withstand severe acidic soil conditions. Cassava does not need substantial care, has high yield per surface, and has a low cost of production compared with other types of crops[7,8]. The use of cassava as a raw material to produce ethanol can therefore be a mean of financially assisting cassava farmers.

Thailand is a tropical country, and the average temperature during daytime is considerably high, particularly in the summer. As a result of heat circulation during ethanol fermentation by yeast, the temperature in the fermentation tanks increases to levels that inhibit yeast growth, decrease survival rate, and impair ethanol fermentation. Hence, to ferment ethanol, it is essential to use a cooling system that ventilates the heat generated from yeast activity[9–11]. In doing so, the cost of ethanol production inevitably rises.

This study searches for new thermotolerant yeast strains which can produce ethanol at high temperatures, using cassava as the feedstock. Through a combination of cheap substrate and an effective ethanol-producing yeast, this alternative will be a viable option for a fuel source.

MATERIALS AND METHODS

Preparation of materials

Cassavas were washed in fresh water to get rid of sand, then peeled and chipped. The wet chips were sun-dried for 2 days and processed with a blender. The flour was sieved through an 80 mesh screen, and then prepared for analysis of moisture following the Thai Industrial Standards TISI No. 52 number 6.4. The total starch in flour was analysed by modifying methods of AOAC[12] and AACC[13]. This flour was used as the substrate.

The slurry of cassava flour (30% w/v) prepared in distilled water was adjusted to an initial pH of 6.0 before being liquefied by α-amylase (Alphamalt VC 5000; 5250 units/g) at a concentration of 1 g per kg of dry starch and temperature range between 95 and 100 °C for 2 h with agitation. The $CaCl_2$ at the concentration of 1 g per kg of dry starch was added to the slurry for enzyme stabilization. After 2 h, the temperature of the fluid dextrin was reduced to 65 °C, the pH was adjusted to 4.0 with 1 M HCl and the saccharification was then completed by loading amyloglucosidase (AGU 750 units/g) at a concentration of 0.45 g per kg of dry starch. The solution was incubated at 65 °C for 24 h under agitation on a water bath shaker. After the saccharification was completed, the reaction was stopped by boiling for 20 min. Aliquots were sampled for analysis of reducing sugar concentration by Nelson-Somogyi method [14].

Isolation and selection of thermotolerant yeast strains

Yeasts were isolated from the soil in the sugarcane, cassava and pineapple plantations in five provinces of Thailand, namely Chachoengsao, Chonburi, Prachinburi, Ratchaburi, and Kanchanaburi. The isolation was done using the diluted sugarcane blackstrap molasses medium containing 5 g/l reducing sugar, 0.5 g/l $(NH_4)_2SO_4$ and an initial pH of 4.5 with a shaking speed of 160 rpm for 72 h at 25–30 °C. The cultures were purified by cross streaking on agar plates containing the same medium and then were incubated at 25–30 °C for 2–3 days. The purified cultures were kept on YPD agar (yeast extract 5 g/l, peptone 10 g/l, dextrose 10 g/l and agar 20 g/l) slant and maintained at 4 °C.

The selection of thermotolerant yeast strains was performed in 5 ml YPD broth supplemented with 40 ml/l of ethanol. Ethanol was obtained in 16 × 100 ml test tubes, which were soaked in a water bath at various temperatures (37, 40, 45, and 50 °C). The yeast strains could be grown at a temperature range 37–50 °C. They were then examined in cassava starch hydrolysate medium containing 20 g/l reducing sugar, 0.5 g/l $(NH_4)_2SO_4$, having an initial pH of 4.5 and supplemented with 40 ml/l of ethanol. The successful cultures were kept at the 4 °C for further screening.

Screening of thermotolerant yeast strains for ethanol production at 45 °C

The yeast strains could be grown at 45 °C in the cassava starch hydrolysate medium supplemented with 40 ml/l of ethanol. The yeasts were screened in the cassava starch hydrolysate medium composed of 180 g/l reducing sugar, 0.5 g/l $(NH_4)_2SO_4$, with an initial pH 4.5. Inocula were prepared by transferring 1 full loop of the yeast cells to 20 g/l reducing sugar of cassava starch hydrolysate medium containing 0.5 g/l $(NH_4)_2SO_4$, pH 4.5, which was obtained in a 125-ml Erlenmeyer flask containing 50 ml of medium and a shaking speed of 160 rpm at 25–30 °C for 24 h. The inocula were transferred to the 5% rate of the screening medium and were cultivated at 45 °C for 72 h at the shaking speed of 120 rpm. The slop was sampled for the analysis of the ethanol concentration.

Identification of thermotolerant yeast strains

The selected yeast strains were morphologically and physiologically characterized following Ref. 15. Yeast strains growing on YM agar for 24 h were morphologically studied in terms of cells, both true mycelium and pseudo-mycelium formation by using the Dalmau plate technique. Ballistospores and ascospores formation as well as the growth examination of yeast strains were undertaken at various temperatures: 30, 35, 37, 40, 45, and 50 °C.

The yeast strains were also identified by the molecular taxonomy following the method of Kurtzman and Robnette [16]. Briefly, the yeast cells were suspended in sterile distilled water and kept in a frosted container, then heated for 10 min at boiling point and put back in the same frosted container.

DNA was extracted and used to amplify 26S rDNA by PCR technique. The PCR product was purified with a PCR purification kit and purified again by the ethanol/EDTA method before it was calculated for base taxonomy of D1/D2 domain of a large subunit of ribosomal DNA (26S rDNA) with a DNA sequencer. The obtained nucleotides were compared for similarities in the GenBank database.

Optimization of ethanol fermentation in a shaking flask

The fermentation medium in a 500-ml Erlenmeyer flask was performed in triplicate using 200 ml of basal cassava starch hydrolysate medium, composed of different reducing sugar concentrations of 160, 180, and 200 g/l with added 0.5 g/l $(NH_4)_2SO_4$; initial pH was adjusted to 4.5 with 1 M HCl and 1 M NaOH. Inoculum size was 5%. The flasks were incubated at 45 °C with the shaking speed of 120 rpm. Samples were taken every 6–12 h for a period of 72 h. The growth, as well as sugar and ethanol concentration, was measured.

The other nutrient compositions such as nitrogen source, phosphorus source, magnesium source, and

yeast extract were also studied. The culture medium was supplemented with 0.5 or 1 g/l of $(NH_4)_2SO_4$, KH_2PO_4, or $MgSO_4$ and the results compared to that without supplementation. Finally, the yeast extract was supplemented at 1, 2 g/l, or without. The best ethanol fermentation conditions were selected for further study.

Ethanol fermentation in a fermenter

The inoculum media placed in 20 g/l reducing sugar, 0.5 g/l $(NH_4)_2SO_4$, pH 4.5 was autoclaved at 121 °C for 15 min. A loopful of yeast cells was added into 100 ml inoculum media in a 250-ml Erlenmeyer flask and the culture was shaken at a speed of 160 rpm at room temperature for 24 h.

The composition of the nutrients in the main media for fermentation was selected from the optimized condition in the shaking flask. The main medium was autoclaved at 121 °C for 20 min and then 200 ml of the inoculum was transferred (5% v/v) into 4 l of main media and cultivated at 45 °C for 72 h.

Batch fermentation was carried out in a 7 l jar fermenter with 4 l of working volume. The fermentation was performed in duplicate at 45 °C for 72 h with agitation speed of 300 rpm and aeration rate of 0.2 vvm throughout the fermentation[9]. The slop was sampled for monitoring the growth, sugar consumption, and ethanol concentration every 4–12 h for a period of 72 h.

Analytical methods

The growth of yeast was monitored by measuring the optical density at the wavelength of 660 nm using a spectrophotometer (Shimadzu; UV-pharmaspec 1700). After washing the yeast cells with 0.1 M HCl twice, they were centrifuged at 4000 rpm for 5 min. The cell pellet was then resuspended in 0.1 M EDTA pH 7.

The ethanol concentration was analysed by gas chromatography using the Varian Star 3600 GC apparatus equipped with an autosampler 8200 injector, a flame ionization detector and a capillary DB-WAX column coated at 70 °C. The injector and the detector were maintained at 200 °C. Nitrogen gas was used for carrier samples with flow rate 4 ml/min. The internal standard used was *n*-propanol[17]. The ethanol concentration was calculated from the ratio of area of ethanol and propanol and then compared with the standard. The ethanol productivity was calculated from the final ethanol concentration subtracting the initial ethanol concentration, and dividing by the fermentation time. The fermentation yield depended on the initial sugar concentration[18].

The sugar concentration was measured using Somogyi-Nelson method[14]. High sugar concentration was diluted with distilled water until the optical density at the wavelength of 520 nm was in the range of 0.1–1.0. The sugar concentration of the samples was compared with the glucose standard.

RESULTS

Isolation and selection of thermotolerant yeast strains

In this study, thermotolerant yeast strains were isolated from soil samples that are associated with sugarcane, cassava, and pineapple fields. The samples were then enriched in the diluted sugarcane blackstrap molasses supplemented with 40 ml/l of ethanol and incubated on a rotary shaker at 25–28 °C for 72 h. A total of 267 yeast strains obtained were used to determine the growth at elevated temperatures by using the YM broth as a substrate. Among these, 2 strains were capable of growing at 50 °C and 169 strains survived and grew at a temperature of 45 °C, while 96 strains could only endure a maximum of 40 °C. The yeast strains that were capable of growing at 45 °C were cultivated in the basal cassava medium containing 20 g/l reducing sugar, 0.5 g/l $(NH_4)_2SO_4$ and the initial pH of 4.5, then 40 ml/l of ethanol was added in the medium and incubated at 45 °C in a water bath. The results revealed that 33 strains grew satisfactorily even at 45 °C.

Screening of thermotolerant yeast strain by ethanol production at 45 °C

The ethanol production of 33 strains was performed in the basal cassava medium containing 180 g/l reducing sugar, 0.5 g/l $(NH_4)_2SO_4$ and an initial pH of 4.5 by cultivation at 45 °C for 72 h. All of them were able to produce ethanol at various concentrations (Table 1).

The 4 strains that produced ethanol higher than 20 g/l were PBB511-1, TM512-2, CPY514-1, and TG514-2. Among these, PBB511-1 produced ethanol at a slightly greater concentration than TM512-2 (Table 1).

Identification of the thermotolerant yeast strains

The strains PBB511-1, TM512-2, CPY514-1, and TG514-2 were identified morphologically and physiologically following a taxonomic key[15]. The colonies of the 4 yeast strains growing on YM agar turned out murky white, low embossed, and with margins smooth to lobed. The growth in the YM broth at room temperature showed sediments of cells at the bottom of the tube. Moreover, the colour of the media became

Table 1 Ethanol concentration of thermotolerant yeast strains cultured in shaking flask at 45 °C.

Strains	EtOH (g/l)	Strains	EtOH (g/l)	Strains	EtOH (g/l)
CPY514-1	23.59	CSK516-3	6.98	PR516-2	15.33
CPK514-1	15.27	CSK5110-2	4.67	TM512-1	16.22
CPK514-2	13.20	CSK5110-3	19.34	TM512-2	26.20
CPK514-3	14.26	BB516-1	4.28	TG513-1	2.59
CPK514-4	14.73	BB516-2	14.17	TG513-2	12.23
CPK518-3	12.60	BB519-1	2.95	TG513-3	4.44
CSK511-1	19.82	BB519-2	3.41	TG514-1	13.89
CSK511-2	17.22	BB519-3	4.17	TG514-2	22.51
CSK511-3	16.98	PBB511-1	26.22	TG518-3	3.58
CSK516-1	7.19	PBB511-2	15.02	PM515-1	8.62
CSK516-2	7.47	PR516-1	14.48	PM515-2	16.28

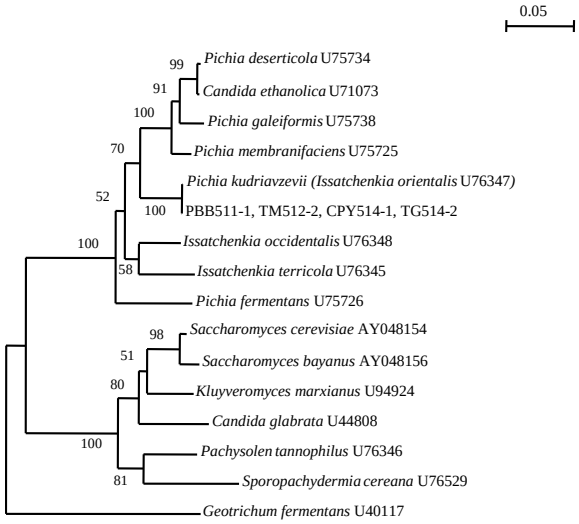

Fig. 1 Phylogenetic tree of PBB511-1, TM512-2, CPY514-1, and TG514-2.

faded with a thin film formed at the surface. When the cells were studied under the light microscope, they were ovoid to elongated in shape. A pseudo-mycelium was observed with moderately branched appearance under the coverglass after 7 days at room temperature. No ascospores or ballistospores were found. The results also showed that the 4 yeast strains could grow at a maximum temperature of 45 °C.

The molecular taxonomy of the strains was done using the nucleotide sequences of D1/D2 domain of the large subunit ribosomal DNA. Their sequences were the same as that of *Pichia kudriavzevii* (*Issatchenkia orientalis*). Hence the strains PBB511-1, TM512-2, CPY514-1, and TG514-2 were identified as *P. kudriavzevii*. The phylogenetic tree of the strains is shown in Fig. 1.

Optimization of ethanol fermentation in shaking flask cultivation at 45 °C

The strain PBB511-1, found to produce the greatest ethanol concentration, was chosen for optimization in various conditions. The effect of sugar concentration on ethanol fermentation of this strain was investigated in a basal cassava media composed of 160, 180, or 200 g/l reducing sugar. The results revealed that the appropriate basal cassava medium for ethanol production was the medium composed of 180 g/l reducing sugar (Fig. 2a). At 48 h, the ethanol concentration was 23.9 g/l, the productivity 0.5 (g/l)/h, and the yield 26% of the theoretical yield. The increase of sugar concentration to 200 g/l reducing sugar had not been accrued for the ethanol production. At 48 h, the ethanol concentration was 23.2 g/l, the productivity 0.48 (g/l)/h, and the yield 23% of the theoretical yield. The decrease of ethanol concentration involved several factors such as high osmotic pressure conditions and high temperatures[19]. On the other hand, the decrease of sugar concentration to 160 g/l reduced the ethanol concentration. The ethanol produced was 15.2 g/l, with a productivity of 0.32 (g/l)/h, and a yield of 19% of theoretical yield (Table 2, Fig. 3a). The decrease of sugar concentration was probably due to the fact that most of the reducing sugar was used for growth. Hence there was less reducing sugar available to be transformed into ethanol.

The effect of $(NH_4)_2SO_4$ as the N-source was examined at concentrations of 0, 0.5, and 1.0 g/l in a basal cassava medium composed of 180 g/l reducing sugar. A greater ethanol production was obtained in the medium containing nitrogen at 0.5 g/l (Fig. 2b). At 48 h, the ethanol produced was 25.8 g/l with a productivity of 0.54 (g/l)/h and a yield of 28% of theoretical yield. The fermentation without N-source produced ethanol at 14.0 g/l, a productivity of 0.29 (g/l)/h and a yield of 15% of theoretical yield, while the fermen-

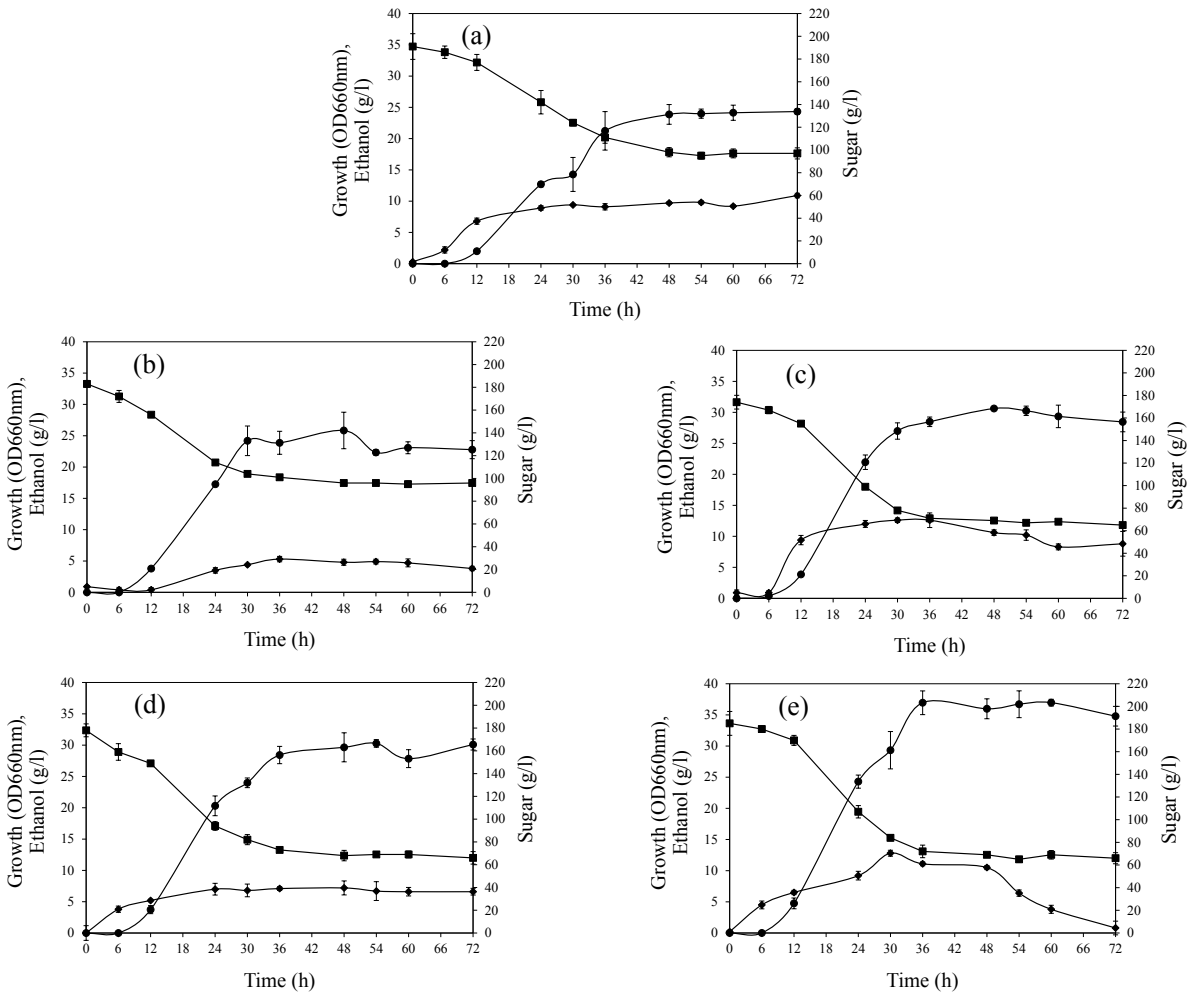

Fig. 2 Growth (diamonds), ethanol production (circles), and sugar consumption (squares) of yeast strain PBB511-1 in cassava medium composed of (a) 180 g/l reducing sugar, (b) 180 g/l reducing sugar, 0.5 g/l $(NH_4)_2SO_4$, (c) 180 g/l reducing sugar, 0.5 g/l $(NH_4)_2SO_4$, 0.5 g/l KH_2PO_4, (d) 180 g/l reducing sugar, 0.5 g/l $(NH_4)_2SO_4$, 0.5 g/l KH_2PO_4, 0.5 g/l $MgSO_4 \cdot 7\,H_2O$, and (e) 180 g/l reducing sugar, 0.5 g/l $(NH_4)_2SO_4$, 0.5 g/l KH_2PO_4, 0.5 g/l $MgSO_4 \cdot 7\,H_2O$, 1 g/l yeast extract. All cultures had initial pH 4.5 and were agitated at 120 rpm at 45 °C.

tation in the presence of 1.0 g/l $(NH_4)_2SO_4$ produced ethanol at 18.4 g/l, with a productivity of 0.38 (g/l)/h and a yield of 20% of theoretical yield (Table 2, Fig. 3b). Nitrogen is important to the yeast cells and can affect their growth rate. A decrease or increase in nitrogen can result in a slow growth rate for yeast cells[20,21]. In the first 48 h, the growth of yeast cells without N-source was very slow ($OD_{660\,nm} = 4.4$), while with 0.5 and 1.0 g/l of $(NH_4)_2SO_4$ the yeast cells had growing rates that was almost the same at $OD_{660\,nm}$ of 4.8 and 5.5, respectively. In the case of sugar, the fermentation with an addition of 0.5 g/l $(NH_4)_2SO_4$, sugar had a dramatic decrease in the first 24 h, and remained at 96 g/l as it approached 72 h of fermentation. On the other hand, the fermentation with an added 1.0 g/l $(NH_4)_2SO_4$ and no N-source had remaining sugar concentrations of 105 g/l and 116 g/l, respectively.

The influence of the concentration of phosphorus on ethanol production was studied as follows: concentration of sugar 180 g/l, 0.5 g/l $(NH_4)_2SO_4$, with a P-source in KH_2PO_4 concentrated format of 0, 0.5, and 1.0 g/l. The study within 48 h revealed that the concentration of 0.5 g/l KH_2PO_4 could produce ethanol higher that both 0 and 1.0 g/l (Fig. 3c). The fermentation with 0.5 g/l KH_2PO_4 produced 30.6 g/l, with a productivity of 0.64 (g/l)/h, and a yield of 33% of theoretical yield (Fig. 2c). The fermentation

Table 2 Ethanol production by PBB511-1 yeast strain in cassava medium containing different concentrations of reducing sugar and nutrient elements (initial pH 4.5, speed 120 rpm, 45 °C).

Condition	Ethanol production after 48 h			Ethanol production maximum			
	ethanol (g/l)	productivity ((g/l)/h)	yield (% of theoretical)	time (h)	ethanol (g/l)	productivity ((g/l)/h)	yield (% of theoretical)
Sugars (g/l)							
160	15.2	0.32	19	54	17.3	0.32	21
180	23.9	0.50	26	72	24.3	0.34	26
200	23.2	0.48	23	54	24.6	0.46	24
$(NH_4)_2SO_4$ (g/l)							
0	14.0	0.29	15	54	15.1	0.28	16
0.5	25.8	0.54	28	48	25.8	0.54	28
1.0	18.4	0.38	20	36	19.1	0.53	21
KH_2PO_4 (g/l)							
0	25.9	0.54	28	48	25.9	0.54	28
0.5	30.6	0.64	33	48	30.6	0.64	33
1.0	27.9	0.58	30	48	27.9	0.58	30
$MgSO_4 \cdot 7\,H_2O$ (g/l)							
0	25.3	0.53	28	48	25.3	0.53	28
0.5	29.7	0.62	32	54	30.3	0.56	33
1.0	27.8	0.58	30	48	27.8	0.58	30
Yeast extract (g/l)							
0	32.2	0.67	36	48	32.2	0.67	36
1.0	36.0	0.75	39	36	37.0	1.03	40
2.0	35.7	0.74	39	48	35.7	0.74	39

without the additional P-source and 1.0 g/l KH_2PO_4 produced ethanol at 25.90 and 27.9 g/l, with productivities of 0.54 and 0.58 (g/l)/h, and yields of 28% and 30% of theoretical yield, respectively (Table 2).

At the end of 72 h, the concentration of ethanol of the three cases and the growth rate of yeast cells decreased, since the rate of ethanol production was quite high in the initial 30–54 h of fermentation (data not shown). In the case of using sugar, the medium containing 0.5 g/l KH_2PO_4 took more sugar than in other conditions, and at the end of fermentation there was 65 g/l sugar remaining, while sugar remained at 75 g/l and 73 g/l for the medium without P-source and with 1.0 g/l KH_2PO_4, respectively.

The influence of magnesium on ethanol production under the basal cassava medium was studied using reducing sugar at 180 g/l, 0.5 g/l $(NH_4)_2SO_4$, and 0.5 g/l KH_2PO_4, with three concentrations of the Mg-source ($MgSO_4 \cdot 7\,H_2O$) tested: 0, 0.5, and 1.0 g/l. The results showed that fermentation with 0.5 g/l $MgSO_4 \cdot 7\,H_2O$ produced higher ethanol than in the other cases (Fig. 3d). At 48 h of fermentation, the culture with 0.5 g/l $MgSO_4 \cdot 7\,H_2O$ produced 29.7 g/l ethanol, with a productivity of 0.62 (g/l)/h and a yield of 32% of theoretical yield. On the other hand, the fermentation without Mg-source and with 1.0 g/l $MgSO_4 \cdot 7\,H_2O$ produced 25.4 and 27.8 g/l ethanol, with productivities of 0.53 and 0.58 (g/l)/h, and yields of 28% and 30% of theoretical yield, respectively (Table 2).

In each of these three cases, there was marginal ethanol accumulation at the end of 72 h, but the yeast growth was comparable, growing very quickly during the first 24 h and remaining constant until the conclusion of the experiment. As for sugar consumption, fermentation with 0.5 g/l $MgSO_4 \cdot 7\,H_2O$ added was found to consume more sugar than other conditions, and the remaining sugar was 66 g/l (Fig. 2d). On the other hand, fermentation without Mg-source and with 1.0 g/l $MgSO_4 \cdot 7\,H_2O$ added had 75 and 74 g/l of sugar remaining, respectively.

The influence of a yeast extract to ethanol production using a basal cassava medium was studied using medium containing 180 g/l reducing sugar, 0.5 g/l $(NH_4)_2SO_4$, 0.5 g/l KH_2PO_4, and 0.5 g/l $MgSO_4 \cdot 7\,H_2O$. The production of ethanol due to fermentation when adding 1.0 g/l yeast extract was greater than both with 2.0 g/l yeast extract and without yeast extract (Fig. 3e, Fig. 2e). At 48 h, the fermentation with 1.0 g/l yeast extract produced 36.0 g/l ethanol, with a productivity of 0.75 (g/l)/h and yield of 39% of theoretical yield. The fermentation in medium

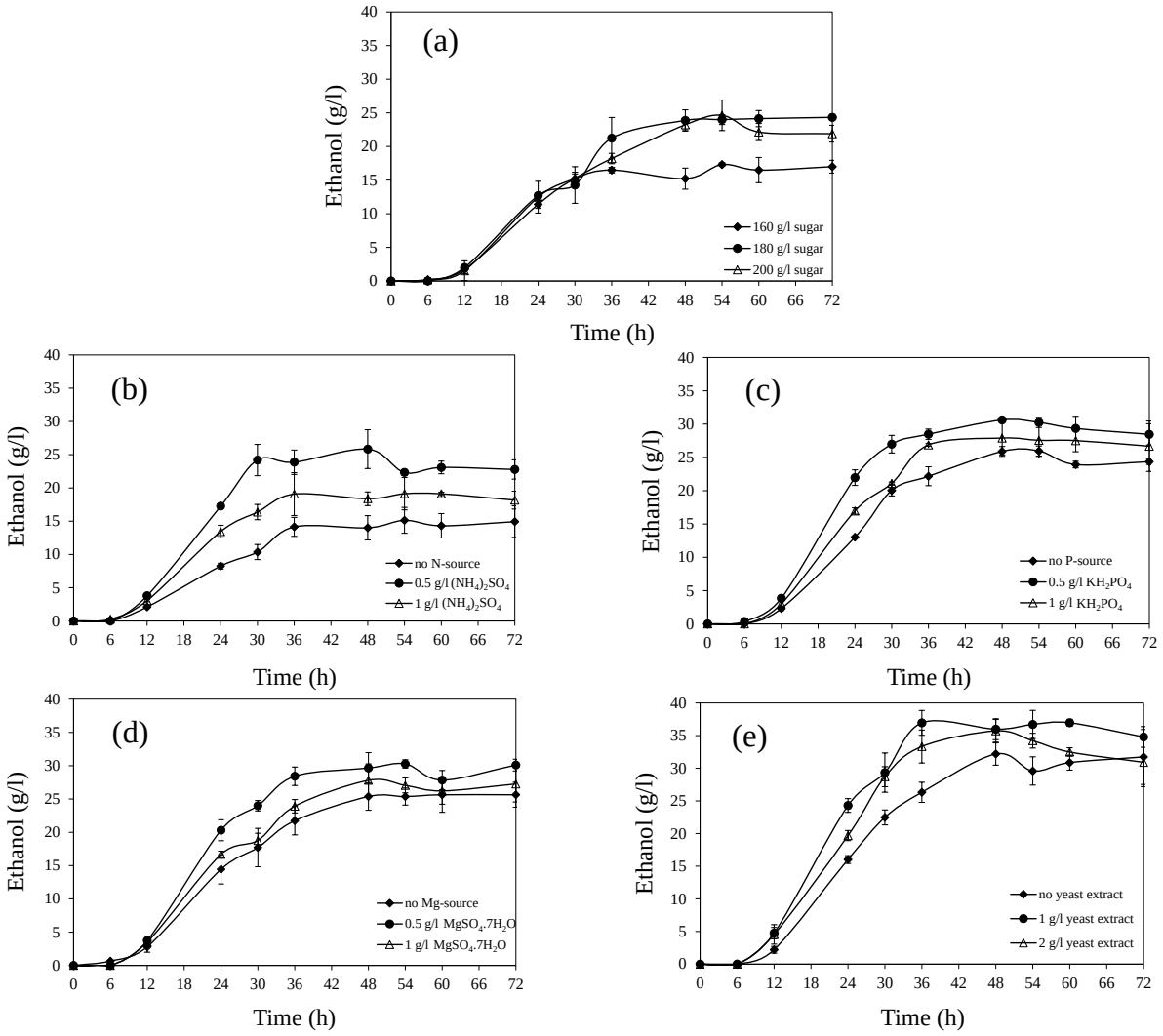

Fig. 3 Comparison of ethanol fermentation under different conditions: (a) sugar concentration, (b) N-source, (c) P-source, (d) Mg-source, and (e) yeast extract.

with 2.0 g/l yeast extract produced 35.7 g/l ethanol, with a productivity of 0.74 (g/l)/h, and a yield of 39% of theoretical yield. On the other hand, the fermentation without yeast extract produced 32.8 g/l ethanol, with a productivity of 0.67 (g/l)/h and a yield of 35% of theoretical yield (Table 2). It was found that ethanol concentration in these three cases decreased at 72 h of the fermentation: the fermentation with 1.0 g/l yeast extract, 2.0 g/l yeast extract, and without yeast extract added produced 34.5 g/l, 30.1 g/l, and 31.7 g/l ethanol, respectively. During the first 30 h of fermentation, the yeast growth in all three conditions was very fast, but after 30 h the growth decreased. This implied that some of the strains did not survive and may have resulted in an increase in ethanol concentration during the period between 36 and 48 h and an increase in the fermentation at 45 °C.

Considering the sugar consumption of these three cases, it was found that during the first 36 h of the fermentation, sugar concentration rapidly decreased and remained constant as the fermentation approached 72 h. The fermentations with 1.0 g/l yeast extract, 2.0 g/l yeast extract, and without yeast extract added were found to contain 66, 70, and 75 g/l sugar, respectively.

Ethanol fermentation was performed at 45 °C by shaking flask of this yeast strain using 180 g/l pure glucose as the substrate. The results showed the highest ethanol production of 45.1 g/l at 36 h, with a productivity of 1.25 (g/l)/h and a yield of 49% of

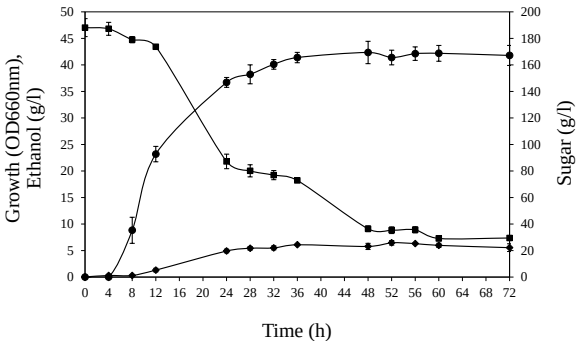

Fig. 4 Ethanol production at 45 °C of *P. kudriavzevii* PBB511-1 by batch fermentation with agitation speed of 300 rpm and an aeration rate of 0.2 vvm throughout the fermentation. Growth (diamonds), ethanol production (circles), and sugar consumption (squares).

theoretical yield. After the fermentation finished, the remaining glucose concentration was 31 g/l. The study also looked at cassava starch containing reducing sugar 180 g/l as a substrate. The yeast produced 37.0 g/l of ethanol at 36 h, with a productivity of 1.03 (g/l)/h and a yield of 40% of theoretical yield. This means that the cassava starch was incompletely converted to glucose. These results imply however that this strain could be able to produce ethanol at high temperature.

From previous studies, the optimized conditions for this strain involved fermentation at room temperature. The results revealed that the ethanol produced was 58.8 g/l at 48 h, with a productivity of 1.23 (g/l)/h and a yield of 64% of theoretical yield. After 72 h, the remaining reducing sugar was 4.7 g/l. Hence this strain could produce ethanol at both room temperature and high temperature.

Ethanol fermentation in a fermenter

The ethanol fermentation of the strain PBB511-1 by batch fermentation at 45 °C in basal cassava starch medium composed of 180 g/l reducing sugar, 0.5 g/l $(NH_4)_2SO_4$, 0.5 g/l KH_2PO_4, 0.5 g/l $MgSO_4 \cdot 7\,H_2O$, 1.0 g/l yeast extract, with an initial pH of 4.5 was performed at agitation speed of 300 rpm and an aeration rate of 0.2 vvm throughout the fermentation. The results showed that the maximal ethanol concentration was 42.4 g/l at 48 h, with a productivity of 0.88 (g/l)/h and a yield of 46% of theoretical yield. The ethanol concentration rapidly increased during the period between 4 and 24 h, after which there was only a slight increase in ethanol, and the sugar consumption sharply decreased during the 12–24 h period. After this interval, there was a slow ethanol concentration decline. After the fermentation period of 72 h, the sugar remaining in the fermenter was 29 g/l. The growth of yeast gradually increased between 12 and 24 h and remained steady until 72 h (Fig. 4).

DISCUSSION AND CONCLUSIONS

Samples of 267 yeast strains were analysed to identify effective thermotolerant yeast strains. A total of 33 strains were found to be able to produce ethanol from cassava starch hydrolysate at 45 °C, and 4 of them showed a superb capability in producing ethanol. PBB511-1, TM512-2, CPY514-1, and TG514-2 produced ethanol at 26.2, 26.2, 23.6, and 22.5 g/l, respectively. The 4 strains were later classified as *P. kudriavzevii* (*I. orientalis*), not as well-known as *Saccharomyces cerevisiae* or *Kluyveromyces marxianus*, which are often used to produce ethanol. *K. marxianus*, a thermotolerant yeast, has been shown to be able to produce ethanol at temperatures up to 45 °C[9,22,23]. *P. kudriavzevii* has been suggested for use in ethanol fermentation since it could be easily found in rice flour ('loog-pang' in terms of Thai traditional fermentation starter)[24]. In addition, there was a study on a yeast growth (*I. orientalis* DY252) under an air support based batch and fed-batch to use as probiotic source[25]. Furthermore, this yeast was recently studied as a fuel source for biodiesel and bioethanol[26–28].

This study was aimed to discover an appropriate condition for producing ethanol by the yeast strain PBB511-1 using a cassava starch hydrolysate medium at 45 °C, initial pH 4.5. The result revealed that cassava starch medium with a concentrated reducing sugar of 180 g/l, 0.5 g/l $(NH_4)_2SO_4$, 0.5 g/l KH_2PO_4, 0.5 g/l $MgSO_4 \cdot 7\,H_2O$, and 1.0 g/l yeast extract was effective in producing ethanol. Within the first 36 h, it can produce ethanol at 37.0 g/l, with a productivity of 1.03 (g/l)/h and a yield of 40% of theoretical yield. Recently, Yuangsaard et al[29] reported that *P. kudriavzevii* DMKU 3-ET15 (isolated from traditional fermented pork sausage) could produce ethanol in a cassava starch hydrolysate medium pH 5.0 composed of 18% glucose, 0.05% $(NH_4)_2SO_4$, 0.09% yeast extract, 0.05% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7\,H_2O$. At 45 °C, the yeast produced ethanol 4% (w/v), with productivity of 1.27 (g/l)/h and yield of 42% of the theoretical yield. Recently a study by Dhaliwal et al[28] reported the isolation of *P. kudriavzevii* from sugarcane juice. At 40 °C, the yeast produced 71.9 g/l of ethanol and a productivity of 4.0 (g/l)/h from sugarcane juice composed of 14% (w/v) sucrose, 2% (w/v) glucose, and 1% (w/v) fructose. From the aforementioned reason, *P. kudriavzevii* can be

a candidate to produce ethanol at high temperature similar to *K. marxianus*.

The batch fermentation was also subjected to 45 °C growth conditions with agitation speed of 300 rpm and an aeration rate of 0.2 vvm throughout the fermentation period. The results revealed that ethanol was produced at the peak level of 42.4 g/l at 48 h, with a productivity of 0.88 (g/l)/h and a yield 46% of the theoretical yield. Under the same conditions, *K. marxianus* DMKU 3-1042 at 37 °C produced 6% (w/v) of ethanol, with a productivity of 1.3 (g/l)/h and a yield of 57% of theoretical yield[9]. When *P. kudriavzevii* DMKU 3-ET15 was exposed to 40 °C with an agitation speed of 300 rpm and an aeration rate of 0.1 vvm throughout the fermentation, it produced a final ethanol of 7% (w/v) after 33 h, with a productivity of 2.23 (g/l)/h and a yield of 80% of the theoretical yield[29]. Hence the study confirms that the yeast strain PBB511-1 can be a candidate for ethanol production at high temperatures.

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