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Research Article

Ethanol production at high temperature from cassava pulp by a newly isolated *Kluyveromyces marxianus* strain, TISTR 5925

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Abstract: *Kluyveromyces marxianus* TISTR 5925, isolated from rotten fruit in Thailand, can ferment at pH 3 at temperatures between 42 and 45 °C. Bioethanol production from cassava pulp using the simultaneous saccharification and fermentation (SSF) process was evaluated and compared with the separated hydrolysis and fermentation (SHF) process using *K. marxianus* TISTR 5925. The ethanol concentrations obtained from the SSF process were higher than those from the SHF process. The optimum conditions for ethanol production were investigated by response surface methodology (RSM) based on a five level central composite design involving the following variables: enzyme dilution (times), temperature (°C) and fermentation time (h). Cassava pulp was pretreated by boiling for 10 min, treated with a mixture of enzymes (cellulase, pectinase, α-amylase and glucoamylase), then fermented by *K. marxianus* TISTR 5925. Data obtained from the RSM were subjected to analysis of variance and fit to a second order polynomial equation. At optimum enzyme dilution (0.1 times), temperature (41 °C) and fermentation time (27 h), the maximum obtained concentration of ethanol was 5.0% (w/v), which is very close to the predicted ethanol concentration of 5.3% (w/v).

Keywords: Ethanol; Thermotolerant; Response surface methodology; Cassava pulp

1. Introduction

The production of ethanol using crops is increasing rapidly and is causing considerable concern that this may limit the supply of food in the future. Lignocellulosic biomass such as agricultural residue is an alternate, inexpensive source of fermentable sugars [1]. Cassava (*Manihot esculenta*) is

an important crop, ranking sixth in the world's food crops in tropical and sub-tropical Africa, Asia and Latin America [2]. Thailand is one of the largest cassava producers, exporting around 16 million tons annually. Forty percent of this cassava root is used as raw material for the production of tapioca starch and at least 1 million tons of pulp within these residues is wasted annually [3,4]. Cassava pulp contains up to 50-60% starch on a dry basis, together with cellulosic fiber [4]. Cassava pulp is mainly used for animal feed and fertilizer, although it could also be used to produce fuel ethanol, obviating the need to compete with food crops. There are many studies regarding ethanol production from cassava pulp [5,6,7].

Saccharomyces cerevisiae is generally used for ethanol production. The growth temperature range for *S. cerevisiae* optimal for fuel ethanol fermentation is 30–35 °C [8]. However, the optimal growth temperature range for conventional strains of *S. cerevisiae* is relatively low (25 to 30 °C). In tropical countries, ethanol fermentation at high temperature is a key requirement. The advantages of rapid fermentation at high temperature are not only the decreased risk of contamination, but also a reduction in cooling costs [9]. Ethanol production at high temperature has been reported for several species such as *Candida tropicalis* and *Kluyveromyces marxianus* [6,9,10,11,12]. *C. tropicalis* of cassava pulp was used in a simultaneous saccharification and fermentation (SSF) process at 40 °C, but the reported ethanol concentration was low (1.4% w/v) [6].

In the current study, a thermotolerant yeast, *K. marxianus,* was screened from a natural resource in Thailand for efficient ethanol production at high temperature. The isolated strain can grow at up to 45 °C and ferment at a pH below 3. Response surface methodology (RSM) was used for efficient ethanol production at high temperature from cassava pulp. To find the optimal conditions for a multivariable system [13], RSM and central composite design (CCD) were employed to evaluate the effects of enzyme dilution, temperature, and fermentation time on ethanol production. The optimum factors were obtained following statistical analysis of the data.

2. Materials and Methods

2.1. Isolation and identification of the microorganism

Yeast samples were isolated from various natural sources of rotten fruit in Thailand. First, the samples were inoculated in broth containing 1% yeast extract, 2% polypeptone, 10% glucose, 0.0015% sodium azide and 0.002% chloramphenicol, and incubated at 42 °C for 24 h. 200 μL of each yeast culture was spread on plates of the same medium plus 2% agar. Colonies of yeast with different appearance were picked, inoculated into liquid medium, and incubated at 42 °C for 2 days. Ethanol production from each culture was determined by gas chromatography (GC 4000; GL Science; Tokyo, Japan) using a glass column (Chromosorb 103, 60/80 mesh , ID 3 ∅ x 3m; Shinwa Chemical Industries Ltd., Kyoto, Japan) and flame ionization detector under the following conditions: split flow 50 mL/min; air flow 250 mL/min; N_2 carrier flow 30 mL/min; temperature of injector, column, and detector: 185, 250, and 250 °C , respectively. N-propanol was used as an internal standard.

The D1/D2 domain sequence of the large subunit (LSU) of rDNA and the internal transcribed spacer (ITS) was determined from the polymerase chain reaction (PCR) products of the genomic DNA. The D1/D2 domain of the LSU rDNA was amplified by PCR using the forward primer NL-1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and the reverse primer NL-4 (5'-GGT 3.3.2 CCG TGT TTC AAG ACG G-3'). The temperature cycles for the PCR reactions were: initial

denaturizing for 10 min at 94 °C, followed by 36 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min. The ITS sequencing primers were the forward-strand primer ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and the reverse-strand primer ITS4 (5'TCC TCC GCT TAT TGA TAT GC-3'). PCR conditions were as above, except for annealing at 60 °C. The sequences were compared using a BLAST homology search [14]. The selected *K. marxianus* strain, TISTR 5925, was deposited at the Thailand Institute of Scientific and Technological Research (TISTR).

2.2. Characterization of growth and ethanol fermentation of the selected strain

TISTR 5925 was characterized by its growth and ethanol fermentation at different temperatures (30, 42 and 45 °C) and pH (from pH 3 to pH 6). *K. marxianus* TISTR 5925 was inoculated into 10% YPD (1% yeast extract, 2% polypeptone and 10% glucose) broth at 30 °C, cultured at 100 rpm for 24 h, and used as the yeast pre-culture inoculant in subsequent experiments. The inoculant was added to fresh medium to an optical density (600 nm) of 0.2 and the cultures were incubated on a rotary shaker (100 rpm) at 30, 37, 42, 44, 45, 46, 48 and 50 °C. Ethanol growth fermentation by TISTR 5925 was carried out at pH 2, 3, 4, 5 and 6, at 30, 42 and 45 °C, in the same manner. Ethanol concentration was determined 5 h after inoculation. Ethanol yield was calculated from the amount of sugar consumed and the maximum level of ethanol obtained after 48 h fermentation. The ethanol productivity was defined as the final ethanol concentration divided by the batch time at 6 h. Ethanol yield was defined as the ratio of ethanol concentration (g/L) and substrate consumed (g/L) (Y_{SE}) compared with the maximum theoretical ethanol yield of 0.51 g/g. The percentage of the theoretical yield was calculated by:

$$\text{Percentage of yield} = 100 \times Y_{SE} / 0.51$$

2.3. Effect of pretreatment of cassava pulp on saccharification

Cassava pulp was obtained from Sanguan Wongse Starch Industries in Nakhon Ratchasima province, Thailand. Dried pulp was prepared by heating at 60 °C for 48 h, then it was ground and sieved through a 0.5-mm mesh screen (ZM-100; Retsch GmbH; Haan, Germany). To determine optimum pretreatment methods for ethanol production, the following methods were examined. The enzyme dosage for saccharification of cassava pulp was modified from Rattanachomsri et al. [6].

1. No treatment: Distilled water (5 mL) was added to 1 g of dry cassava pulp, as a control.
2. Boiling treatment: Distilled water (5 mL) was added to 1 g of dry cassava pulp and boiled at 100 °C for 10 min.
3. Autoclave treatment: Distilled water (5 mL) was added to 1 g of dry cassava pulp and autoclaved at 120 °C, 15 psi for 10 min.
4. Sonication treatment: Distilled water (5 mL) was added to 1 g of dry cassava pulp. Sonication was performed at a maximum 10 power burst for 60 s, using a Misonix astrason® 3000 (Wakenyaku Co., Ltd.; Kyoto, Japan).

A portion of each sample was subjected to enzymatic hydrolysis using 100 U cellulase (Celluclast® 1.5 L; Novozyme A/S; Bagsvaerd, Denmark) per gram dry pulp and 100U pectinase (Pectinex Ultra SP-L; Novozyme A/S; Bagsvaerd, Denmark) per gram dry pulp. The remainder of each sample was not enzymatically treated (controls). All samples were incubated for 24 h at 50 °C, then liquefaction was carried out at 80 °C for 1 h with 55 U α-amylase (Termamyl® 120 L;

Novozyme A/S; Bagsvaerd, Denmark) per gram dry pulp. Next, 20 U glucoamylase (AMG 300, Novozyme A/S, Bagsvaerd, Denmark) per gram dry pulp [6] was added and the samples were incubated at 50 °C for 24 h. Released glucose was measured by high performance liquid chromatography (HPLC; LC10A; Shimadzu Corp.; Kyoto, Japan) using refractive index detection (RID) and a CARBOSep CHO-682 LEAD column (Transgenomic Inc.; Omaha, NE) at 80 °C. The mobile phase was deionized water (0.4 mL/min).

2.4. Ethanol fermentation using separate hydrolysis and fermentation, and simultaneous saccharification and fermentation

The separated hydrolysis and fermentation (SHF) process involves hydrolysing 20% (w/v) cassava pulp using 100 U cellulase and 100 U pectinase per gram of cassava pulp at 50 °C at100 rpm for 24 h, followed by 55 U α-amylase per gram of cassava pulp at 80 °C for 1 h and 20 U glucoamylase per gram of cassava pulp at 50 °C at 100 rpm for 24 h. The pulp hydrolysates were supplemented with 1% yeast extract and 2% polypeptone as nitrogen sources. In the SSF process, the same levels of enzymes and nitrogen sources as used in the SHF process were combined and cultured. TISTR 5925 strain was incubated in 10% YPD at 30 °C as a pre-culture. The pre-culture was inoculated into SHF and the SSF medium to an optical density of approximately 12 (600 nm). The culture was incubated at 42 and 45 °C with rotary shaking at 100 rpm for 48 h. Samples were removed at various time intervals and analyzed for ethanol by GC, for sugar concentration by HPLC, and for reducing sugar by the Somogyi-Nelson method [15]. -

2.5. Optimization of the simultaneous saccharification and fermentation process

The effects of enzyme concentration, temperature and fermentation time using the SSF process were further studied using statistical experimental design. After pretreatment by boiling, the slurries were cooled to 30 °C and cellulase, pectinase, α-amylase, glucoamylase were added to the cassava medium. The yeast pre-culture was used to inoculate the media to an optical density (OD 600 nm) of approximately 2. Ethanol production by *K. marxianus* TISTR 5925 from cassava pulp was optimized by RSM because this is a useful statistical tool for studying and predicting the interactions among several factors that are varied in a number of experiments [16]. The SSF process was performed according to the CCD, which is composed of three factors (enzyme dilution, temperature, and fermentation time) and five levels of estimated values (−1.686, −1, 0, +1, +1,686) (detailed in Table 3) with rotary shaking at 100 rpm. Concentrations of the ethanol and residual sugar produced were determined by GC and HPLC, respectively, as described above. The fermentation efficiency was calculated based on the total available sugar. The theoretical yield of ethanol was calculated as 0.51. Enzyme dilution (X1, times), temperature (X2, °C) and fermentation time (X3, h) were selected as the independent variables. The ethanol concentration (Y %, w/v) was used as the dependent output variable. The variable X_i was coded as x_i: i means varied factors (enzyme dilution, temperature, and fermentation time)

$$x_i = (X_i - X_c) / \Delta x_i \qquad i=1, 2, 3, 4 \qquad (1)$$

where x_i is the dimensionless value of an independent variable, X_i the real value of the independent variable, X_c is the real value of the independent variable at the center point of 0 among five levels of estimated values (−1.686, −1, 0, +1, +1.686) and Δx_i is the step change of variable i [17]. The true

values of the variables are given in Table 1 and the enzyme dosages are shown in Table 2. The CCD for 3 factors (k = 3) contained a total of 17 experiments including three replications at the center point. The independent variables and their levels are presented in Table 3. STATISTICA for Windows (Release 5.0, Stasoft, Tulsa, OK) was used for the experimental design.

Table 1. Process variables used in the central composite design (K = 3) with actual factor levels corresponding to coded factor levels.

Factor	Code[a]	Actual factor level at coded factor levels of:				
		-1.682[b]	-1	0	1	+1.682
Enzymes dilution (times)*	X1	0.006	0.01	0.1	1	1.692
Temperature (°C)	X2	32	35	40	45	48
Fermentation time (h)	X3	4	12	24	36	44

[a]Code level limits based on preliminary investigations and also reflective of what was done in practice.

[b]Level based on the CCD

*Enzyme dosages are given in Table 2

Table 2. Enzyme dosages based on dry weight.

Type of enzyme	Enzyme dosage (X1)				
	0.006	0.01	0.1	1	1.692
Cellulase (U/g dry pulp)	0.06	0.1	1	10	16.92
Pectinase (U/g dry pulp)	0.6	1	10	100	169.2
α-Amylase (U/g dry pulp)	0.33	0.55	5.5	55	93.1
Glucoamylase (U/g dry pulp)	0.12	0.2	2	20	33.8

Table 3. Process variables used in the CCD (K = 3) with actual factor levels corresponding to coded factor levels.

Treatment	Coded variable level			Y (Ethanol, % w/v)	
	X1 Enzyme dilution	X2 Temperature, °C	X3 Fermentation Time, h	Observed	Predicted
1	-1 (0.01)	-1 (35)	-1 (12)	2.74	3.54
2	-1 (0.01)	-1 (35)	+1 (36)	4.76	7.07
3	-1 (0.01)	+1 (45)	-1 (12)	2.94	2.41
4	-1 (0.01)	+1 (45)	+1 (36)	4.53	4.27
5	+1 (1)	-1 (35)	-1 (12)	2.88	2.5
6	+1 (1)	-1 (35)	+1 (36)	7.44	7.01
7	+1 (1)	+1 45)	-1 (12)	1.12	1.03
8	+1 (1)	+1 (45)	+1 (36)	2.44	3.86

9	-1.682 (0.006)	0 (40)	0 (24)	3.71	4.83
10	+1.682 (1.692)	0 (40)	0 (24)	2.98	3.23
11	0 (0.1)	-1.682 (32)	0 (24)	5.77	5.10
12	0 (0.1)	+1.682 (48)	0 (24)	1.52	1.91
13	0 (0.1)	0 (40)	-1.682 (4)	1.17	2.46
14	0 (0.1)	0 (40)	+1.682 (44)	6.18	7.11
15	0 (0.1)	0 (40)	0 (24)	5.76	4.78
16	0 (0.1)	0 (40)	0 (24)	6.04	4.78
17	0 (0.1)	0 (40)	0 (24)	5.90	4.78

2.6. Statistical analysis

The experimental data were analyzed according to RSM to fit a second-order polynomial:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X^2_1 + b_{22}X^2_2 + b_{33}X^2_3 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 \qquad (2)$$

where: Y is the ethanol concentration (% w/v); X_1, X_2, X_3 are the independent variables (enzyme dilution, temperature, and fermentation time) (Tables 1 and 3); b_0 is the offset term; b_1, b_2, b_3 are the linear effects; b_{11}, b_{22}, b_{33} are the square effects; and b_{12}, b_{23}, b_{13} are the cross effects of the interaction terms.

3. Results

3.1. Isolation and identification of the microorganism

From the collected samples, 564 yeast colonies were selected and further screened four times on 10% YPD at 42 °C to obtain pure yeast colonies. One strain (TISTR 5925) was identified because it consistently produced ethanol yields higher than 4% (w/v) in four independent trials (data not shown). TISTR 5925 was identified using a conventional taxonomic approach by comparing its morphology, sugar assimilation and the results of a fermentation test (data not shown). The results suggested that strain TISTR 5925 belongs to *K. marxianus*. In addition, the D1D2 domain of the large-subunit and the ITS region showed 98 and 97% homology, respectively, to *K. marxianus*. These results identified TISTR 5925 as *K. marxianus.*

3.2. Characterization of growth and ethanol fermentation of K. marxianus TISTR 5925

The fermentation of glucose to ethanol was tested at various temperatures (30, 37, 42, 44, 45, 46, 48 and 50 °C) and pH levels (2, 3, 4, 5, and 6). The maximum ethanol concentration produced by *K. marxianus* TISTR 5925 at 42 °C was 4.7% (w/v). The productivity of TISTR 5925 was similar at 44 and 45 °C (1.8 and 1.7 g/L/h, respectively) and higher than at 30 and 46 °C (0.8 g/L/h), while the highest productivity was at 37 °C (2.7 g/L/h). Ethanol yields of this strain at 30-46 °C were in the range 89.7-95.9% (Fig. 1). TISTR 5925 strain exhibited the same levels and rates of ethanol fermentation at high temperature as strain NCYC2791 (data not shown).

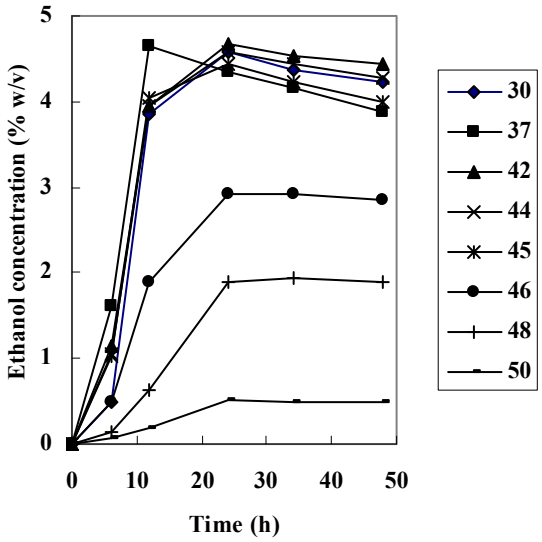

Figure 1. Ethanol production by *K. marxianus* TISTR 5925 in 10%YPD (yeast extract, polypeptone and D-glucose) from 30 to 50 °C. All experiments were repeated at least twice.

The effect of the initial medium pH on fermentation by *K. marxianus* TISTR 5925 and NCYC2791 was investigated at 30, 42 and 45 °C. Both strains can ferment well at all pH values tested, except for pH 2 at 30 °C. When the temperature was increased to 42 and 45 °C, only *K. marxianus* TISTR 5925 was able to ferment at pH 3 (Table 4). This result indicates that *K. marxianus* TISTR 5925 can ferment at low pH better than NCYC2791.

Table 4. Ethanol yield of *K. marxianus* TISTR 5925 and NCYC2791 in 10%YPD at various pH values and temperatures.

Ethanol yield (%)	30 °C		42 °C		45 °C	
	TISTR 5925	NCYC 2791	TISTR 5925	NCYC 2791	TISTR 5925	NCYC 2791
pH 2	36.3	0.0	0.0	0.0	0.0	0.0
pH 3	102.8	97.0	79.8	0.0	68.8	0.0
pH 4	92.5	92.7	83.1	86.6	80.2	79.2
pH 5	92.5	95.4	87.3	91.7	88.7	74.8
pH 6	87.4	91.3	84.4	86.7	82.1	84.7

3.3. Effect of pretreatment on saccharification of cassava pulp

To determine the optimal pretreatment methods for ethanol production from cassava pulp, various methods in combination with cellulase and pectinase mixtures were examined—namely, no treatment, boiling, autoclaving, and sonication. Sonication proved effective at releasing starch from cassava pulp in the absence of added enzymes. However, the level of released sugar was as high as 95-98% of total sugar following all pretreatments when enzymes were added, and the addition of the

enzyme mixture clearly improved the saccharification reaction (Fig. 2). Boiling in combination with enzyme addition was selected as the pretreatment for the following experiment because the investment cost for infrastructure required for boiling is lower than for sonication.

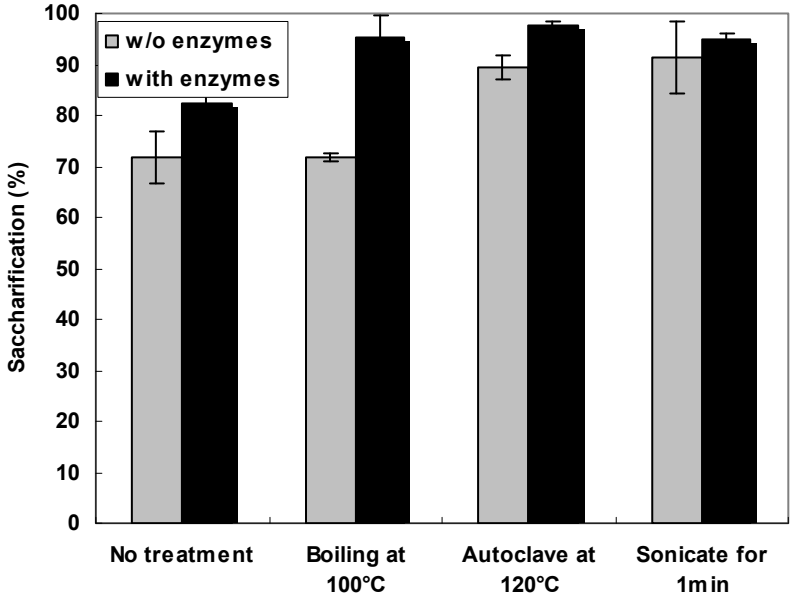

Figure 2. Saccharification of cassava pulp after pretreatment using various methods. (Enzymes = cellulase and pectinase)

3.4. Ethanol fermentation when hydrolysis and fermentation are performed separately, and when saccharification and fermentation are performed simultaneously

A typical SSF batch conversion was performed with all enzymes (cellulase, pectinase, α-amylase and glucoamylase) at 42 and 45 °C on 20% dry cassava pulp. The SSF process was essentially complete after 24 h. The final ethanol concentration was 5.6 ± 0.1% (w/v) at 42 °C and 4.6 ± 0.0% (w/v) at 45 °C, and the productivity of ethanol was 3.6 ± 0.3 g/L/h at 42 °C and 4.5 ± 0.1 g/L/h at 5 h at 45 °C (Fig. 3). On the other hand, the SHF process produced a maximum ethanol concentration of 5.0 ± 0.2% (w/v) at 42 °C and 3.5 ± 0.0% (w/v) at 45 °C after 24 h. The productivity of ethanol was 2.8 ± 0.3 g/L/h for 5 h at 42 °C and 3.7 ± 01 g/L/h for 5 h at 45 °C.

3.5. Optimization of the simultaneous saccharification and fermentation process

Table 1 shows the three independent variables (enzyme dilution, temperature and fermentation time) at different coded and actual levels of the variables employed in the design matrix. Five levels of the CCD matrix in X1-X3 (X1: enzyme dilution, X2: temperature, X3: fermentation time) and the experimental variables (Y: ethanol concentration, % w/v) are listed in Table 3. Seventeen experiments based on the CCD were carried out with different combinations of variables; the results are also presented in Table 3. The quantity of ethanol produced by *K. marxianus* TISTR 5925 ranged from 1.1 to 7.4% (w/v). The regression equation coefficients were calculated and their data fit to a

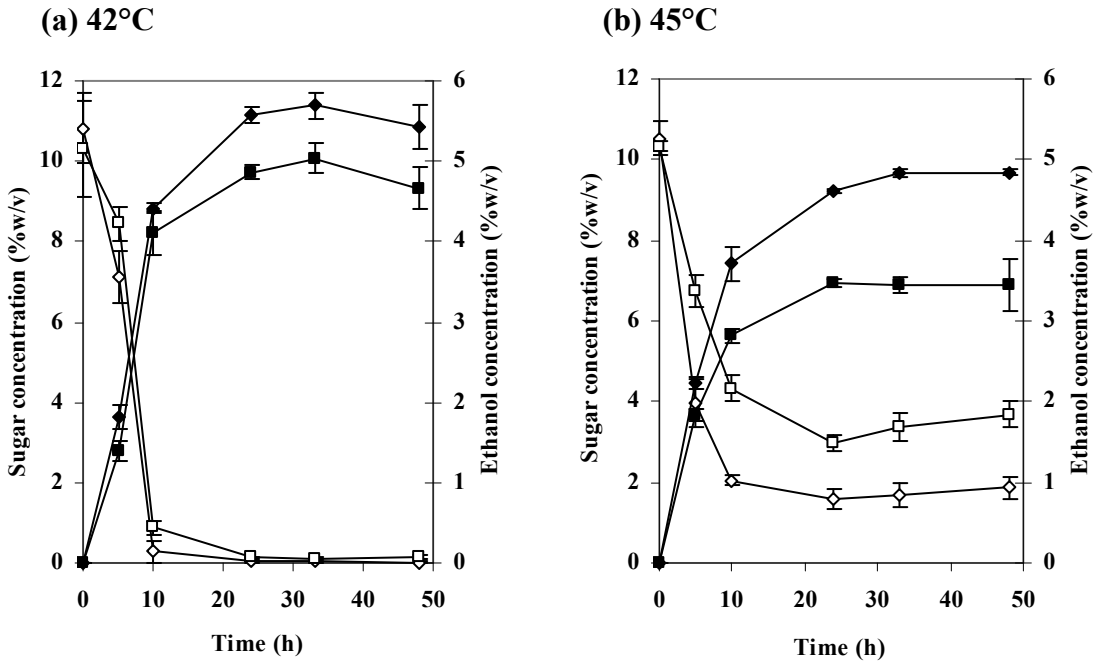

Figure 3. Comparison of ethanol production and sugar consumption during simultaneous saccharification and fermentation (SSF) and separated hydrolysis and fermentation (SHF) at 42 °C (a) and 45 °C (b) by *Kluyveromyces. marxianus* TISTR 5925. ♦ ethanol concentration during SSF; ■ ethanol concentration during SHF; ◊ sugar concentration during SSF; □ sugar concentration during SHF. All experiments were repeated at least three times.

second-order polynomial equation. The response Y (ethanol concentration, % w/v) by TISTR 5925 could be expressed in terms of regression Equation (3). The actual ethanol concentrations from experiments and the predicted ethanol concentrations from Equation (3) are given in Table 3.

$$Y = (1.572X_2) + (0.392X_3) - (0.311X^2_1) - (0.02X^2_2) - (0.035X_1X_2) - (0.041X_1X_3) - (0.007X_2X_3) - 28.740 \quad (3)$$

Table 5 shows the F-test analysis of variance for various models. This model, Eq. (3), was significant with a p-value of ≤ 0.05. In addition, the lack of fit was not significant (F_{LOF} =0.2316 < $F_{0.05, (1,8)}$ = 5.3000), indicating that the model, Eq. (3), was adequate to fit the experimental data (Table 5).

Table 5. Analysis of variance for the fitted quadratic polynomial model for ethanol production.

Source	Sum of Squares	df	Mean square	F value	*p*-value
Model	45.3910	7	6.4840	3.6400	0.038
Linear	37.5550	2	18.7780	11.0140	0.001
Quadratic	17.3720	2	8.6860	2.7600	0.098
Interaction	23.9680	3	7.9890	2.7730	0.084
Residual error	16.0320	9	1.7810	-	-

Lack of fit	0.4510	1	0.4510	0.2316	-
Pure error	15.5810	8	1.9476	-	-
Total	61.4230	16	-	-	-

$R^2 = 0.739$

The response surface plots according to the regression model are shown in Fig. 4. The shape of the contour plots indicates that the mutual interactions among the independent variables were significant. From the response surface plots, the optimal values of the independent variables could be determined; the interactions among independent variables are shown in Fig. 4. The orientation of the contour plots between enzyme dilution and temperature indicated that enzyme dilution and temperature had a significant effect on the bioconversion of starch to ethanol (Fig. 4a). Although the fermentation time showed no significant interaction with the other variables, the long fermentation time resulted in increased ethanol concentration (Fig. 4b).

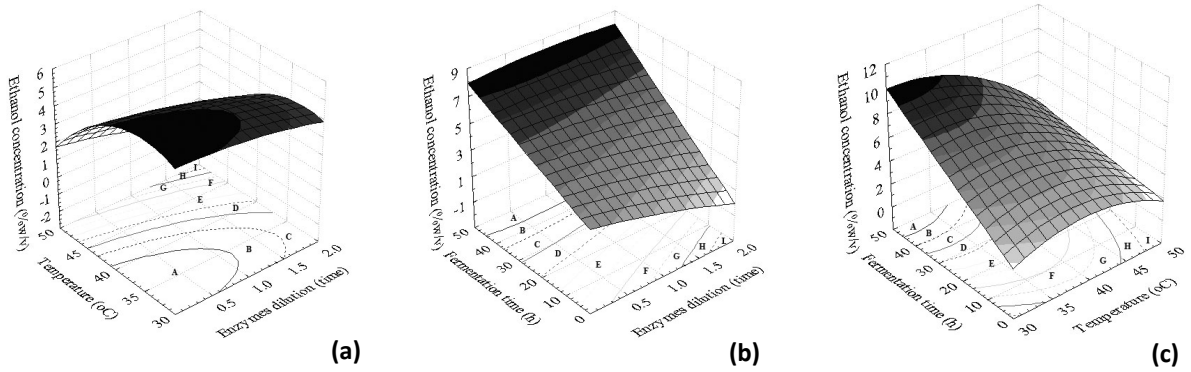

Figure 4. Surface and contour plots showing the relative effect of pairs of factors on ethanol production: (a) effect of enzyme dilution and temperature, (b) effect of enzymes dilution and fermentation time, (c) effect of temperature and fermentation time. Upper case letters indicate different zones of enzyme dilution, temperature and fermentation time for production of ethanol. 'A' indicates the optimum zone.

Generally, the optimum temperature for enzymatic starch saccharification is around 50 °C, whereas the optimal temperature for fermentation by *S. cerevisiae* is around 30 °C. This 20 degree difference between saccarification and fermentation indicates that the selected temperature should not be so low that catalysis of saccharification cannot occur, or so high that yeast cannot grow. The response surface plots indicated that the maximum ethanol concentration was within the enzyme dilution range 0.006–1.25 and the temperature range of 32–41 °C (Fig. 4b and 4c). Consequently, the optimal factors for saccaharification and fermentation were 0.1 for enzyme dilution (cellulase 1 U, pectinase 10 U, α-amylase 5.5 U and glucoamylase 2 U/g of dry cassava pulp), 41 °C for temperature and 28 h for the fermentation time. Under these conditions, the maximum ethanol concentration was predicted to be 5.3% (w/v). This prediction was tested by experimentation (Table 6). The maximum ethanol concentration and ethanol yield were 5.0% (w/v) and 81.4%, respectively, which are very similar to the predicted values in the RSM (Table 6). The good correlation among the four results confirmed the validity of the response model and the model was proven to be adequate.

Table 6. Confirmation of the experiments.

Enzyme dilution (times)	Temperature (°C)	Fermentation time (h)	Ethanol concentration (%, w/v)	
			Predicted	Experimental
0.006	44	35	4.24	4.65
0.1	44	35	4.66	4.63
0.1	41	28	5.29	5.00
0.1	38	20	4.71	4.58

4. Discussion

The thermotolerant yeast, *K. marxianus*, produces ethanol efficiently at high temperature and thus holds promise for commercial ethanol production in tropical countries such as Thailand. *K. marxianus* TISTR 5925 was isolated from rotten fruit in Thailand and can grow at temperatures of up to 45 °C (Fig. 1). This thermotolerant yeast can produce ethanol with higher yield at 30–45 °C even under acidic conditions (pH 3) than type strain NCYC2791 (Table 4).

Pretreatment damages the cellulose structure of cassava pulp, thus facilitating starch release from starch granules. Boiling cassava pulp followed by cellulase and pectinase treatment was selected as the pretreatment of choice in this study because infrastructure costs for heating are lower than for sonication and autoclaving, which are also effective pretreatments. Although pectinase did not directly promote starch hydrolysis, it helped to reduce the viscosity of the starch from cassava pulp [18]. A similar result was reported by Srichuwong et al. [19], who produced ethanol from very high gravity (VHG) potato mash using *S. cerevisiae* NBRC0224. When a combination of pectinase, cellulase and hemicellulase was used to pretreat VHG potato mash, the viscosity was noticeably reduced and the ethanol yield was 89.7% based on the theoretical yield. The SSF process involves the hydrolysis of polymers into glucose and the conversion of glucose to ethanol in the same vessel. The primary advantage of the SSF process compared to the SHF process is the cost savings resulting from the reduced number of reactor vessels needed, the increased rate of hydrolysis due to decreased product inhibition, the reduction in fermentation time, and decreased capital cost [20]. Comparing the SSF and SHF processes, the entire SSF process (33 h at 42 °C) was shorter than that of SHF (97 h at 42 °C). In SHF at 42 °C, hydrolysis was for 49 h, and fermentation was for 48 h (Fig. 3a and b). The times were almost the same at 45°C: hydrolysis was for 49 h, and fermentation was for 24 h (Fig. 3a and b). The maximum ethanol concentration and productivity using SSF were higher than the SHF process at 42 °C or 45 °C. Ethanol productivity using the SSF process at 45 °C was faster than using the SHF process. The final ethanol concentration using the SSF process at 42 °C was also higher than in the SHF process. Consequently, the SSF process is superior to the SHF process for ethanol production from cassava pulp.

Optimization of the saccharification and fermentation conditions is important to maximize ethanol production. Statistical methods such as response surface methodology (RSM) have been widely used to optimize the process conditions [19,17,21]. For example, the optimization of pH, temperature and substrate concentration on ethanol yield from pretreated tapioca flour was performed using RSM [17]. In the current study, the factors for saccaharification and fermentation

were optimized by RSM to be 0.1 for enzyme dilution (cellulase 1 U, pectinase 10 U, α-amylase 5.5 U and glucoamylase 2 U/g of dry cassava pulp), 41 °C for temperature, and 28 h fermentation time. These conditions provided 5.3% (w/v) for the predicted ethanol content and 5.0% (w/v) (Table. 6) for the experimentally-obtained ethanol content. These results show that the model provided by Eq. (3) is useful. The concentration of enzymes used for these high temperature fermentations is lower than that used by Kosugi et al. [5]. In this earlier paper, cassava pulp was hydrolyzed to glucose for further ethanol fermentation by *S. cerevisiae* K7. This fermentation consumed cellulase (3 U/g dry pulp), α-amylase (300 U/g dry pulp) and glucoamylase (100 U/g dry pulp) at 30 ° for 5-7 days. The ethanol yield in the current study was 81.4%, which is higher than the yield (61%) reported earlier [5]. The results identified using RSM in the current paper provide the optimum conditions for ethanol production from cassava pulp.

5. Conclusion

This study examined ethanol production from cassava pulp using *K. marxianus* TISTR 5925, which was isolated from natural sources in Thailand. *K. marxianus* TISTR 5925 exhibited high performance in fermentation at high temperature, producing 5.6 ± 0.1% and 4.6 ± 0.0% ethanol at 42 and 45 °C, respectively, from 20% (w/v) cassava pulp using the SSF process.

Moreover, RSM was used as a statistical tool to optimize the pretreatment and fermentation factors (enzyme dose, temperature and fermentation time). The SSF conditions for cassava pulp were identified as: enzyme dilution, 0.1 times (cellulase 1 U, pectinase 10 U, α-amylase 5.5 U and glucoamylase 2 U/g of dry cassava pulp), temperature, 41 °C and fermentation time, 28 h. The predicted high level of ethanol concentration (5.3%) and high yield (83.6%) were similar to the obtained experimental values of ethanol concentration (5.0%) and high yield (81.43%). These results indicate that RSM holds promise for the large scale optimization of ethanol production.

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Conflict of interest

The authors declare that there are no conflicts of interest related to this study.

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