ETHANOL PRODUCTION FROM CASSAVA BAGASSE BY CO-CULTURE OF *SACCHAROMYCES CEREVISIAE* AND *SPATHASPORA ARBORARIAE*

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ABSTRACT

Co-culture fermentation of cassava bagasse was studied by using *Saccharomyces cerevisiae* (*S. cerevisiae*) TISTR 5606 and *Spathaspora arborariae* (*S. arborariae)* ATCC® MYA 4684, in order to improve the fermentation efficiency with the increasing of ethanol yield. Glucose was hydrolyzed after enzymatic pretreatment of cassava bagasse, yielding 55.95 g/l glucose. However, xylose and inhibitors were not observed .Factors affecting ethanol production from cassava bagasse were optimized via response surface methodology (RSM) with Central Composite Design (CCD). The fermentation conditions that gave the maximum ethanol yield and concentration were the co-culture of *S. cerevisiae* and *S. arborariae* at ratio of 1:1 and 32.7 ^{o}C for 1 day. The ethanol concentration of 28.16 g/l was obtained and yield of ethanol production was 0.5.

KEYWORD: Ethanol fermentation, Cassava bagasse, Saccharomyces cerevisiae, Spathaspora arborariae, Co-culture fermentation

1. Introduction

Energy usage including fuel supply, transportations and industries has played important roles to human routines for long ago. Currently, fossil-fuel, petroleum, coal, and natural gas which obtained from geological investigation are widely used as energy resources. However, these are nonrenewable energy, means when it is exhausted and cannot be established or substituted in a short time. Renewing of natural resources will need long time, perhaps it needed more than million years to restore in a limited amount. In contrast, renewable energy can be used infinitely because it can be substituted by itself in a short time, called green or

clean energy because of its non-pollution releasing. Renewable energy comes from solar power, wind power, hydropower, wave power, biomass, and geothermal energy. In order to solve efficiently the problem of a lack of energy in the future, we are interested in the ethanol production which is a familiar renewable energy for human life. Ethanol is used as fuel, beverage, and solvent in many industries. Moreover, ethanol can be produced simply from raw materials that contain glucose. These raw materials can be divided into 3 groups; (1) starch e.g. cassava, rice and corn, (2) sugar e.g. sugarcane and molasses and (3) lignocellulose material e.g. sugarcane bagasse, strew and corn cob. The lignocellulose consists of cellulose, hemicellulose and lignin [1].

Cassava bagasse or cassava pulp was selected as a substrate in this study because the cassava is an economic crop of Thailand. In addition, Thailand has been the third ranking of the world of the largest exporter and the first largest cassava exporter for a long time [2]. Normally, cassava bagasse that can be used for raising animals is by-product from tapioca production. Cassava bagasse consists of the average carbohydrate of 40-64 %, fiber of 21-51, the average of protein of 0.3-1.6, fat of 0.5-1.1, humidity of 5-11 %, and ash of 0.7-1.5 % [3] depending on its species, farming, and efficiency of starch extraction process. The major component of cassava bagasse are carbohydrate and fiber which are used as substrate for ethanol production.

Co-culture fermentation between *S. cerevisiae* and *S. arborariae* may enhance ethanol production because *S. arborariae* can use both of pentoses and hexoses. On the other hand, *S. cerevisiae* can use only hexoses. Fernandada Cunha-Pereira who found that ethanol production by co-culture fermentation between *S. cerevisiae* and *S. arborariae* of rice hulls hydrolysates was observed to be greater than single culture [4].

The aim of this study is to optimize ethanol production from cassava bagasse by using co-culture fermentation with *Saccharomyces cerevisiae* TISTR 5606 (*S. cerevisiae*) and *Spathaspora arborariae* ATCC®MYA 4684 (*S. arborariae)*. Enzymatic pretreatment of cassava bagasse was investigated to study the effects of pretreatment methods on the ethanol yield.

2. Experiments

2.1 Materials

Cassava bagasse was obtained from Eiamheng Tapioca Starch Industry CO., LTD which the tapioca starch company in Nakhon Ratchasima province, Thailand. The size of the average particle of cassava bagasse was less than 16 mesh.

2.2 Microorganisms

In this study, *Saccharomyces cerevisiae* (*S. cerevisiae*) TISTR 5606 and *Spathaspora arborariae* (*S. arborariae*) ATCC®MYA 4684 are two species of microorganisms that were chosen for ethanol fermentation. *S .cerevisiae* was taken from Thailand Institute of Scientific and Technological Research (TISTR), Pathum Thani, Thailand. *S. arborariae* was received from American Type Culture Collection (ATCC), Manassas, VA USA .Culture medium used for the growth of *S. arborariae* and *S. cerevisiae* consist of 1 %w/v glucose, 0.3% w/v yeast extract, 0.3 %w/v malt extract and 0.5 %w/v peptone in distilled water .

2.3 Hydrolysis process

For enzymatic pretreatment, 15 %w/v of cassava bagasse in acetate buffer pH 5 was liquefied by using α-amylase (0.3mg/g) at 90 $^{\circ}$C for 2 hours and then saccharified by using glucoamylase (0.5mg/g) at 60 $^{\circ}$C for 2 hours at 100 rpm . For acid pretreatment, 15%w/v of cassava bagasse was added to 2.5 % (v/v) sulfuric acid in an autoclave at 121 $^{\circ}$C for 20 minutes .The hydrolysate and residues that obtained from cassava bagasse hydrolysis were separated by using filter bag.

2.4 Ethanol fermentation

This part was carried out to study ethanol fermentations by using co-culture of *S. cerevisiae* and *S. arborariae*. The fermentation was done in 250 ml Erlenmeyer flasks that contained with 90 ml culture medium and 10 ml inoculum solution. The fermentation experiments were incubated at 150 rpm for 4 days, while temperature and ratio of microorganism were designed from CCD method. The independent variables were shown in table 1 and all designed conditions of these experiments were also shown in table 2.

2.5 Central composite design (CCD)

Experimental conditions can use CCD to achieve mathematical model for studying the relationship between all variable as well as a quadratic relationship. CCD is designed for 3 levels experiments, which represent in symbols -1, 0 and +1. CCD for 3 variables consists of (i) factorial points (is taken 2 level full factorial to a part of experiment) and (ii) axial points (is an adjustment on a value of a variable) [5].

2.6 Response Surface Methodology(RSM)

For this procedure, it consists of mathematics and statistics techniques which are beneficial for simulation model and problem analysis. It is used for finding appropriate conditions of ethanol fermentation. There are three levels of variables (-1.414, -1, 0, +1, +1.414). For Polynomial or quadratic, it is the model for predicting the most appropriate point of this experimentation (Equation 1). It is also used to analyse the results by using coefficient of determination (R^2) [6].

$$\hat{y} = \beta_0 + \sum_{=i1}^{k} \beta_i x_i + \sum_{=i1}^{k} \beta_{ii} x_i^2 + \sum_{=i1}^{k} \sum_{=j1,\, j<1} \beta_{ij} x_i x_j + \varepsilon \qquad (1)$$

Where $\hat{y}$ is predicted response (ethanol concentration), β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients for the intercept, linearity, square, and interaction, respectively. X_i and X_j are the different interaction coefficients between input factors. ε is error faction. In this study, one equation and two variables of each duration time (two variables for ethanol fermentation were examined). The mathematical relationship among these variables are evaluated by the following polynomial equation:

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$$\hat{y} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 + \varepsilon \qquad (2)$$

Where X_1 and X_2 are the temperature of fermentation and the ratio of *S. cerevisiae* to *S. arborariae*.

2.7 Analytical methods

The amount of starch could be analyzed by using a simplified modification of the AOAC official method 1990 [7]. The method of lipid and fibre analysis [8] and the amount of ash in cassava bagasse [9] were carried out in this work. The moisture content of cassava bagasse could be done by moisture analyzer. The sample broth was taken for analyzing the pH, microbial enumeration, total reducing sugar and ethanol concentration in every 24 hours for 4 days of experiment .The amount of glucose, xylose, furfural, hydroxymethylfurfural (HMF) and ethanol in fermentation broth were analyzed via HPLC instrument, which is equipped with a refractive index (RI) detector and a Bio-Rad HPX-87H column at 60°C. The mobile phase was 5 mM sulfuric acid and was run at a flow rate of 0.6 ml/min.

Table 1 Levels of variables were tested by CCD method.

Independent variables	Levels					Δ
	-1.414	-1	0	1	1.414	
X_1, Temperature (°C)	17.9	20	25	30	32.7	5
X_2, Ratio of *S. cerevisiae* : *S. arborariae*	0.22:0.78	0.3:0.7	0.5:0.5	0.7:0.3	0.78:0.22	0.2

Table 2 CCD design for the optimization and values of observed responses at 24 hours of fermentation.

No.	X_1[a]	X_2[b]	ethanol concentration	
			fermentation	predicted
1	-1	-1	9.89	8.15
2	-1	1	0	0.81
3	1	-1	14.83	17.78
4	1	1	16.11	21.61
5	0	0	17.43	15.77
6	0	0	15.38	15.77
7	0	0	16.71	15.77

Table 2 (continued) CCD design for the optimization and values of observed responses at 24 hours of fermentation.

No.	X_1 [a]	X_2 [b]	ethanol concentration	
			fermentation	predicted
8	0	0	15.1	15.77
9	0	0	15.01	15.77
10	-1.414	0	0	1.65
11	1.414	0	28.16	23.59
12	0	-1.414	13.11	13.01
13	0	1.414	14.29	10.55

[a] X_1, Temperature (°C), [b] X_2, Ratio of microorganism *S. cerevisiae* : *S. arborariae*

3. Results and discussion

3.1 Composition of cassava bagasse

The composition of cassava bagasse is starch (69.96%), fiber (12.88%), lipid (0.39%), ash (4.74%), moisture (5.39%) and others (6.83%). The composition of cassava bagasse shows similar results to the work done by Virunanon et al. [10] who stated that their cassava bagasse consists of starch (75.10%), fiber (9.46%), ash (11.9%) and others (3.61%). The difference of cassava bagasse composition could be possible due to a cultivated area, gene, harvesting period, and flour properties on processing performance.

3.2 Hydrolysis process

Glucose yields were observed when using enzymatic and acid pretreatments of cassava bagasse. The highest yield of glucose was 37.30% ($g_{glucose}/g_{cassava\ bagasse}$), corresponding to the glucose concentration of 55.95 g/l when using 15 %w/v cassava bagasse, α-amylase dose of 0.45 mg/g and glucoamylase dose of 0.8 mg/g, while xylose, furfural and HMF were absent. Moreover, Chen et al. [11] who also study the enzymatic hydrolysis of cassava bagasse. 10% (w/v) cassava bagasse in citrate acid buffer (pH 5.6) was liquefied by 24 mg/g α-amylase at 55 °C for 30 min and saccharified by 65.5 mg/g of glucoamylase at 75 °C and pH 4.0 for 4 hours. Their result yielded the total reducing sugar of 20.0 g/l and total reducing

sugar yield of 0.162 g/g. For acid pretreatment, the maximum glucose yield was 37.95% ($g_{glucose}/g_{cassava\ bagasse}$), corresponding to the glucose concentration of 56.93 g/l when using 15% w/v cassava bagasse and 2.5% v/v H_2SO_4 at 121 °C for 20 minutes. The pretreatment reaction using low acid concentration occurred in the absence of inhibitor. These results showed good agreement with the work done by Phowan et al. [12].

3.3 Ethanol fermentation

The best condition of this experiment was experiment 11, whose conditions were incubation at 32.7 °C, shaking speed of 150 rpm and microbial *S. cerevisiae* : *S. arborariae* ratio of 1:1 as shown in figure 1. The optimum duration time for ethanol production was 24 hours. The yield of ethanol and ethanol concentration were 0.5 and 30.97 g/l, respectively. The ethanol concentration and the predicted concentration from RSM are shown in table 2. The ethanol yield which dropped after 48 hours may be due to low concentration of remaining glucose in the fermentation broth [13]. This finding work showed a good agreement with the results observed from Hickert et al. [14] who studied ethanol fermentation from the hydrolysate of rice hull using co-culture of *S. cerevisiae* and *S. arborariae in an orbital shaker at 30 °C, 180 rpm for 240 h.* In order to investigate ethanol yield observed from different experiments (the temperature and microbial ratio), 13 experiments were carried out. The results were plotted in 3D surface response as shown in figure 2. The relative of linear equations for ethanol yield were presented in equations 3-5 for each fermentation time.

$$\text{At 18h:}\quad Y_1 = 20.3269 - 1.7333X_1 - 21.8999X_2 + 0.0411X_1^2 - 45.1569X_2^2 + 2.4600X_1X_2 \qquad (3)$$

$$\text{At 24h:}\quad Y_2 = -29.3350 + 2.4644X_1 - 19.2670X_2 - 0.0449X_1^2 - 54.9382X_2^2 + 2.7925X_1X_2 \qquad (4)$$

$$\text{At 48h:}\quad Y_3 = -68.5307 + 5.8120X_1 + 16.4937X_2 - 0.1177X_1^2 - 70.5751X_2^2 + 2.0725X_1X_2 \qquad (5)$$

Where Y_1-Y_3 were predicted ethanol concentration of each duration time. X_1 and X_2 are the temperature of fermentation and the ratio of *S. cerevisiae* to *S. arborariae*.

The coefficient of determination (R^2 value) that use to explain the reliability in statistic of the data have the value between 0-1 (with values closer to 1 meaning higher reliability). The R^2 values of Y_1 to Y_3 observed from this work were 0.90, 0.87 and 0.58, respectively.

Therefore predicted ethanol concentration at 18 and 24 hours fermentation time were considered to be a high correlative.

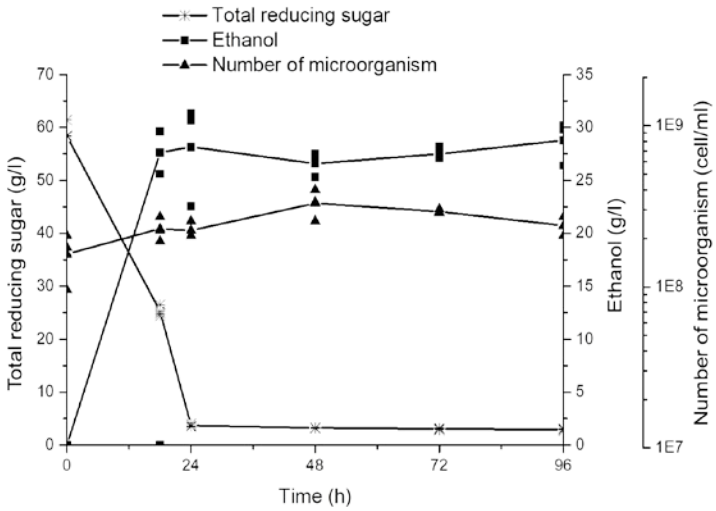

Figure 1 **Ethanol, remaining total reducing sugar and microbial cell concentrations obtained from microbial fermentation of enzymatic hydrolysate of cassava bagasse.**

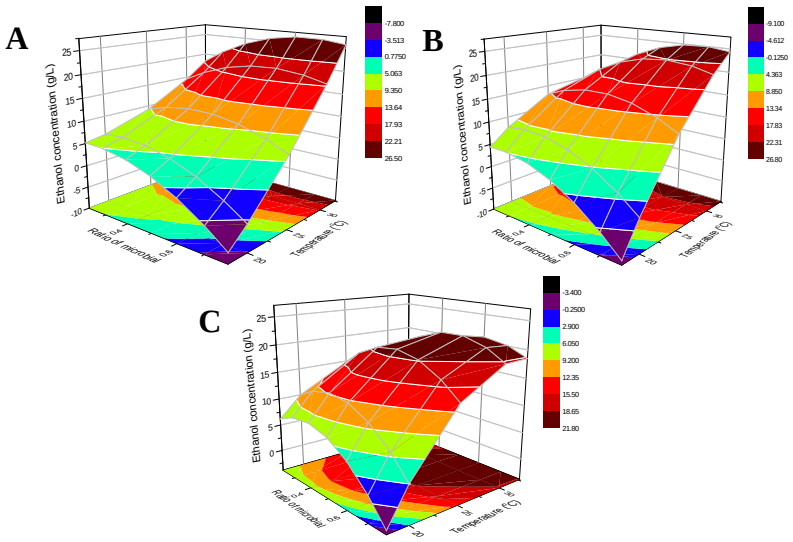

Figure 2 **Response surface curve of ethanol obtained from microbial fermentation of enzymatic hydrolysate of cassava bagasse during fermentation time (A) 18, (B) 24 and (C) 48 hours.**

4. Conclusions

Cassava bagasse can be used as a substrate for ethanol fermentation by using co-culture of *S. cerevisiae* and *S. arborariae* .The optimal condition for ethanol production was 32.7 ^{o}C, 150 rpm and microbial *S. cerevisiae* : *S. arborariae* ratio of 1:1 for 1 day fermentation.

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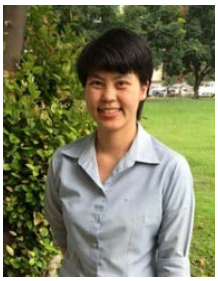

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