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STUDY ON FERMENTATION CONDITIONS FOR BIOETHANOL PRODUCTION FROM COCOA POD HYDROLYSATE

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ABSTRACT

Bioethanol is an environmentally friendly fuel and renewable source in many industrial applications. Cocoa pod is an abundant agro-waste source and can be used for bioethanol production because this lignocellulosic material has high content of cellulose. The aim of this study was to investigate the ability for bioethanol production from cocoa pod hydrolysate. Four strains of Saccharomyces cerevisiae (2.1, B3, D3, D4) were examined for bioethanol production from cocoa pod hydrolysate with the initial inoculation levels at 10^2, 10^4, 10^6, 10^7 cells/mL. The fermenting medium was tested with the reducing sugar concentration in a range of 5.0, 6.0, 7.0, and 8.6% (w/v) and pH value at 4.5, 5.0, 5.5 and 6.0. The fermentation conditions were designed at different temperatures (25, 30, 37°C) and times (3, 5, 7, 9 days). The results showed that the suitable yeast strain for ethanol fermentation was S. cerevisiae D3 with the inoculum level of 10^6 cells/mL. The ethanol concentrations of 4.14-4.19% (w/v) were achieved after 9 days at 30°C from cocoa pod hydrolysate contained 8.6% (w/v) of reducing sugar with initial pH at 5.5, the sugar consumption efficiencies were 97.54-97.56%. These outputs indicated the promising feasibility of bioethanol production from such waste material source of cocoa pods.

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1 INTRODUCTION

In Vietnam, cocoa (*Theobroma cacao*) has been widely grown in thousands of hectares in different ecological regions from the Mekong Delta to the Central Highlands. In 2003, the area of cacao was about 3,000 ha and jumped to 25,000 ha by the end of 2013. It is expected to double by 2015 and the production will be 52,000 tons (Hoa, 2007). Cocoa pod is the by-product from cocoa fruit which was abundant at every harvest season. The cocoa pod makes up about 75% of the total weight of the fruit and becomes an agricultural waste, and a health hazard for the healthy immature cocoa pod, as it harbors cocoa stem borers (Adeleke *et al*., 2012). The cellulose content in the cocoa pods (about 20-40%) is one considerable material for production of fermentable sugar (Duku *et al*., 2011; Thomsen *et al*., 2014).

Saccharomyces cerevisiae is the well known yeast for its fermentation capacity and hence can be employed for alcohol production from various sugar containing materials (van Maris *et al*., 2006; Laluce *et al*., 2012). This yeast is the preferred microorganism for large scale ethanol production

due to its high ethanol yield and productivity (Wallace-Salinas and Gorwa-Grauslund, 2013) and stable fermentation capacity under many stresses (You *et al*., 2003; Stanley *et al*., 2010; Lam *et al*., 2014).

The aim of this study was to investigate the conditions for ethanol fermentation from cocoa pod hydrolysate by using yeast *S. cerevisiae*. The experiments were designed to study the appropriate yeast strains and inoculum levels, the effect of initial reducing sugar concentrations and pH of the hydrolysate, and the fermentation conditions of cocoa pod hydrolysate.

2 MATERIALS AND METHODS

2.1 Strains, materials and medium

Four yeast *S. cerevisiae* strains (2.1, B3, D3, and D4) were selected and maintained at Biotechnology Research and Development Institute, Can Tho University. Cocoa pod hydrolysate was prepared by the method of Phong *et al*. (2015), glucose concentration was 8.6% (w/v). YPD medium (yeast extract 1%, peptone 2%, D-glucose 2%) was used for yeast inoculum preparation.

2.2 Methods

2.2.1 Effect of yeast strains and inoculum levels on ethanol fermentation

To select yeast strain producing the highest ethanol concentration and the corresponding inoculum levels of cells, the experiment was carried out in triplicate with two factors, yeast strains (2.1, B3, D3, and D4) and inoculum levels (10^2, 10^4, 10^6, 10^7 cells/mL). The experiment was randomly performed in 250-mL Erlenmeyer flasks containing 100 mL of cocoa pod hydrolysate medium (treated with $NaHSO_3$ 140 mg/mL in 30 minutes) at 30°C and 150 rpm. The remaining reducing sugar and ethanol concentration were determined after 5 days of fermentation.

2.2.2 Effect of initial reducing sugar concentration and pH on ethanol fermentation

The experiment was randomly arranged with two factors and three replications. The reducing sugar contents of the media were 5.0, 6.0, 7.0, and 8.6% (w/v) and pH values of 4.5, 5.0, 5.5 and 6.0. One hundred milliliters of cocoa pod hydrolysate was inoculated with selected yeast strain and inoculum level as from previous experiment. Reducing sugar concentration of hydrolysate was 8.6% (w/v) and diluted to experimental concentrations. Citric acid 10% (w/v) was used for pH adjustment. The hydrolysate was treated with $NaHSO_3$ 140 mg/mL in 30 minutes. The remaining reducing sugar, pH and ethanol concentration were analyzed after 5-day-incubation at 30°C and 150 rpm.

2.2.3 Effect of temperature and time on the ethanol fermentation

The experiment was randomly carried out in 250-mL Erlenmeyer flasks containing 100 mL of cocoa pod hydrolysate medium in triplicate with two factors, fermentation temperature (25, 30, 37°C) and time (3, 5, 7 and 9 days). The samples were collected and determined the remaining reducing sugar content, pH and ethanol concentration.

2.2.4 Experimental production

The up-scale ethanol production was conducted with 200 mL, 500 mL and 1,000 mL of cocoa pod hydrolysate. The optimum conditions from the previous studies were used for this experimental production.

2.2.5 Analytical methods and statistical analysis

The pH was measured with a digital pH meter (Sartorius PB-20). Total reducing sugar analysis was performed colorimetrically using a dinitrosalicylic acid reagent (Tasun *et al*., 1970). The dinitrosalicylic acid method based on the reaction of alkaline 3,5-dinitro-salicylic acid (DNS) with reducing sugars. Absorbance at 575 nm is proportional to the content of reducing sugar. Ethanol concentration was measured by acid dichromate method (Bennett, 1971). This method based on the complete oxidation of ethanol by dichromate in the presence of sulfuric acid with the formation of acetic acid. The absorbance was measured by using spectrophotometer at 590 nm to determine ethanol. Sugar consumption efficiency (%) was calculated as the actual sugar consumed and expressed as percentage of sugar consumption. Experimental data were statistically analysed using Statgraphics Centurion XV (Manugistics Inc., USA).

3 RESULTS AND DISCUSSION

3.1 Effect of yeast strains and inoculum levels on ethanol fermentation

This experiment was carried out to evaluate the fermentation potential of four *S. cerevisiae* strains (2.1, B3, D3, and D4) and 4 levels of yeast inoculum (10^2, 10^4, 10^6 and 10^7 cells/mL). The result of two-factor interaction was shown in Table 1.

The sugar consumption efficiencies and inoculum levels of 4 strains were tending upwards, ethanol

concentration produced by every treatment did not follow this trend. Generally, when the initial yeast level was 10^2 cells/mL, the 4 strains showed no statistically significant difference. This can be explained that the very-low cell concentration would give the weakness in reproducing and getting ideal density for fermentation (Pham, 2009). The ability of fermenting of 4 strains indicated statistically significant difference when the inoculum levels were at 10^4-10^6 cells/mL. In this range of cell levels, the potential of fermenting of strain D3 was higher than the others with the maximal achieved ethanol of 3.71% (w/v) and the initial cell number of 10^6 cells/mL (Table 1). However, when inoculum level got 10^7 cells/mL, produced ethanol concentration were getting low (2.26-3.39% w/v). This happened when most of sugars had been consumed to increase biomass instead of producing ethanol (Galazzo and Bailey, 1990).

Table 1: Effect of yeast strains and starter density on ethanol fermentation

Factors		Results	
Yeast strain	**Inoculum level (cells/mL)**	**Ethanol concentration (% w/v)**	**Sugar consumption efficiency (%)**
B3	10^2	1.22 ^{j}	92.89 ^{jk}
B3	10^4	1.75 ^{h}	93.98 ^{fgh}
B3	10^6	3.05 ^{c}	94.29 ^{efg}
B3	10^7	2.67 ^{d}	94.61 ^{de}
D3	10^2	1.30 ^{j}	94.32 ^{efg}
D3	10^4	2.15 ^{f}	94.80 ^{cde}
D3	10^6	3.71 ^{a}	95.31 ^{abc}
D3	10^7	3.39 ^{c}	95.49 ^{a}
2.1	10^2	1.19 ^{j}	93.70 ^{hi}
2.1	10^4	1.96 ^{g}	94.57 ^{def}
2.1	10^6	3.37 ^{b}	94.91 ^{bcd}
2.1	10^7	3.10 ^{c}	95.29 ^{ab}
D4	10^2	1.29 ^{j}	92.71 ^{k}
D4	10^4	1.51 ^{i}	93.19 ^{ij}
D4	10^6	2.51 ^{e}	94.02 ^{gh}
D4	10^7	2.26 ^{f}	94.59 ^{def}
Standard error		0.0315	0.0009

Notes: In the same column, the average values with the same letter were not significantly different at the 95% confidence level

3.2 Effect of initial reducing sugar concentration and pH on ethanol fermentation

This experiment was carried out to evaluate the fermentation ability of strain D3 when the initial reducing sugar concentrations were 5.0-8.6% (w/v) and initial pHs were 4.5-6.0 (Table 2). The statistical results by every factor showed that the effects of initial reducing sugar concentrations were significantly different at 95% confidence level.

In the examined range, the produced ethanol decreased (from the highest of 3.72% (w/v) to the lowermost of 0.60% (w/v)) as the initial reducing sugar concentrations went down (Table 2). It was indicated that when sugar concentration decreased, the nutrient was not enough for the growth of yeast biomass resulting in the death of yeast caused by nutrient rivalry (Trumbly, 1992). The statistical results by every factor also indicated that the effects of initial reducing sugar concentrations were significantly different (at 95% confidence level). The degree of activity of yeast could be affected by changing pH (Pampulha and Loureiro-Dias, 1989). Changing pH could change charges of substances on cell membrane or osmosis levels of nutrition and direction of fermentation. If pH level was 4.5-5.5 then the fermentation process would be normal. This experiment controlled just the initial pH so it would slightly decrease after a period of fermentation time. The causes of these changes can be the utilization of sugar by yeast for production of CO_2 or organic acids (Pham, 2009).

Table 2: Effect of initial reducing sugar concentration and pH on ethanol fermentation

Factors		Results		
Initial sugar (% w/v)	**Initial pH**	**Ethanol concentration (% w/v)**	**pH**	**Sugar consumption efficiency (%)**
5.0	4.5	1.28 ^{m}	4.19	92.78 ^{g}
5.0	5.0	1.81 ^{h}	4.61	93.14 ^{fg}
5.0	5.5	1.87 ^{h}	5.06	93.33 ^{efg}
5.0	6.0	0.60 ^{n}	5.60	91.61 ^{h}
6.0	4.5	1.61 ^{j}	4.13	93.51 ^{efg}
6.0	5.0	2.38 ^{f}	4.60	93.72 ^{efg}
6.0	5.5	2.71 ^{e}	5.30	94.29 ^{cde}
6.0	6.0	1.38 ^{l}	5.58	93.79 ^{ef}
7.0	4.5	2.04 ^{g}	4.18	94.00 ^{def}
7.0	5.0	2.86 ^{d}	4.70	94.31 ^{cde}
7.0	5.5	3.19 ^{c}	5.31	95.92 ^{ab}
7.0	6.0	1.49 ^{k}	5.72	95.00 ^{bcd}
8.6	4.5	2.36 ^{f}	4.16	95.23 ^{bc}
8.6	5.0	3.43 ^{b}	4.41	95.61 ^{b}
8.6	5.5	3.72 ^{a}	4.68	96.81 ^{a}
8.6	6.0	1.73 ^{i}	5.29	95.09 ^{bc}
Standard error		0.0147	0.0039	0.0017

Notes: In the same column, the average values with the same letter were not significantly different at the 95% confidence level

The combination effects of the initial reducing sugar concentration and pH were shown in Table 2. It was indicated that when reducing sugar concentrations were low (5.0-6.0%) and initial pH was 6.0, the ability of yeast to grow was limited. This led to the lower amount of produced ethanol compared to the other treatments with higher sugar concentrations and optimal initial pH (4.5-5.5). In the treatments that had initial pH of 4.5, the low-level ethanol concentrations were also recorded as they had low reducing sugar concentrations (7.0-8.6% w/v). In summary, the treatments that had both high sugar concentrations and optimal initial pH (5.0-5.5) would give much ethanol. As shown in Table 2, the treatment with 8.62% (w/v) of reducing sugar and pH of 5.5 (ethanol concentration of 3.72% (w/v) was attained) would be chosen for the next experiments.

3.3 Effect of temperature and time on the ethanol fermentation

Temperature is one of the most important factors in ethanol fermentation. Results show that the effect of temperature on fermentation was meaningful at 95% of confidence level (Table 3). The highest ethanol concentration of 4.06% (w/v) was achieved at 30°C while the highest value at 25°C was 3.47% (w/v). At 37°C, the highest ethanol concentration was only 2.93% (w/v). This could be explained that temperature could affect the activity of functional cellular enzymes. Concretely, the optimal temperature for yeast was about 28-32°C (Pham, 2009).

Duration of fermentation was also a factor that could affect fermentation process. In general, the production of ethanol increased as time rose from 3 to 7 days and slightly decreased 2 days later. It was noticed that the fermentation in this experiment was in a closed system and the medium was a liquid; that means there was a proliferative phase followed by a productive phase (Pham, 2009). In the proliferative phase, the nutrition was used for yeast to grow; by that time, ethanol amount was not so much. In the productive phase, the carbon source was used for ethanol production. Because of those, it could be seen that proliferative phase was still fundamental after 5 days (ethanol concentration got 2.96% w/v). Two days later, productive phase was primary and the ethanol concentration was 3.49% (w/v) averagely (Table 3). After 9 days, there was a lethal phase because of the effects of the nutrient starvation and inhibition; in this period, ethanol level went down slightly although sugars were still consumed. This can be explained that when glucose was depleted, cells consume the earlier produced ethanol via gluconeogenesis, and concomitantly they increased their respiration (Orlandi *et al*., 2013). In conclusion, ethanol concentration at 4.06% (w/v) was achieved after 7 days of fermentation at 30°C and the amount of consumed sugar was 97.74%.

Table 3: Effect of temperature and time on the ethanol fermentation

Factors		Results	
Temperature (°C)	Time (days)	Ethanol concentration (% w/v)	Sugar consumption efficiency (%)
25	3	1.40 ^{h}	88.46 ^{j}
25	5	2.98 ^{d}	94.12 ^{f}
25	7	3.47 ^{c}	96.44 ^{e}
25	9	3.40 ^{c}	97.33 ^{c}
30	3	1.62 ^{g}	88.96 ^{i}
30	5	3.68 ^{b}	96.92 ^{d}
30	7	4.06 ^{a}	97.74 ^{b}
30	9	3.98 ^{a}	98.33 ^{a}
37	3	1.36 ^{h}	83.03 ^{k}
37	5	2.22 ^{f}	90.31 ^{h}
37	7	2.93 ^{de}	92.77 ^{g}
37	9	2.86 ^{c}	96.81 ^{d}
Standard error		0.0176	0.0002

Notes: Values in the table were the average values of triplication. In the same column, the average values with the same letter were not significantly different at the 95% confidence level

3.4 Experimental production

In the range of testing volumes, the result in Table 4 shows the correlation between time and fermentation volume. When fermentation volume was 200 mL, the ethanol concentrations produced had no statistically significant difference between 7, 9, and 11 days of fermentation (4.03%, 3.97% and 3.98% (w/v), respectively). In comparison with the ethanol concentration which carried out with 100 mL of cocoa pod hydrolysate, at 7 days of fermentation, the ethanol concentration was 4.06% (w/v) (Table 3) and as shown in Table 4, the ethanol concentration was 4.03% (w/v) with the volume of 200 mL. It can be concluded that, in the range of 100-200 mL of fermentation volume, the fermentation process was almost finished after 7 days because of the limitation of sugars in the medium.

Table 4: Ethanol produced from different fermentation volumes

Factors		Results	
Fermentation volume (mL)	Time (days)	Ethanol concentration (% v/v)	Sugar consumption efficiency (%)
200	7	4.03 ^{b}	97.47 ^{d}
200	9	3.97 ^{b}	97.69 ^{bc}
200	11	3.98 ^{b}	97.70 ^{bc}
500	7	3.51 ^{c}	96.84 ^{e}
500	9	4.14 ^{a}	97.56 ^{cd}
500	11	4.13 ^{a}	97.96 ^{a}
1,000	7	3.20 ^{d}	95.97 ^{f}
1,000	9	4.19 ^{a}	97.54 ^{cd}
1,000	11	4.14 ^{a}	97.85 ^{ab}
Standard error		0.0244	0.0003

Notes: In the same column, the average values with the same letter were not significantly different at the 95% confidence level

When the fermentation volumes were 500 mL and 1,000 mL, ethanol concentrations showed statistically significant difference between 7-and 9-day periods, the ethanol concentrations were 3.20-3.51% (w/v) and 4.14-4.19% (w/v), respectively (Table 4). When the fermentation time increased up to 11 days, the ethanol concentrations were slightly decreased (4.13-4.14% w/v) although the reducing sugar was still consumed. It was indicated that 9 days of fermentation was enough time for the fermentation process. In conclusion, in the range of testing, the increasing of fermentation volumes

should be considered the fermentation time to guarantee enough time for all sugars could be converted to ethanol.

4 CONCLUSIONS

The suitable conditions for ethanol fermentation from cocoa pod hydrolysate by using *S. cerevisiae* D3 were as follows: inoculation level 10^6 cells/mL, pH 5.5 and incubation temperature 30°C. During 9 days of fermentation, the highest ethanol concentrations could reach 4.14-4.19% (w/v) with the sugar consumption efficiencies found at 97.54-97.56%, indicating the promising application of cocoa pod as one of waste material sources for the bioethanol fermentation.

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