Response Surface Methodology for Optimization of Ethanol Production from Cocoa Waste

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Abstract

Cocoa waste (CW) is an inexpensive agro-industrial by-product that is available in large quantities in tropical countries such as Malaysia. The rate of ethanol production can be affected by different parameters involved during fermentation. In this study, response surface methodology (RSM) with the full factorial design was used to obtain optimum conditions for bioethanol production using CW as the substrate. The simultaneous saccharification and fermentation (SSF) performed with an isolated microorganism from locally fermented food tapai ubi and tapai pulut. The effect of four independent variables temperature, CW concentration, inoculum size, and pH was investigated. In the optimized condition temperature of 31.7°C, pH 6.0, inoculum size 10.5%, and CW concentration 0.3 g/L, the highest ethanol production was 9.5 ± 1.1 . ANOVA analysis revealed that temperature and CW concentration had the most significant effects on ethanol production. In addition, ethanol production was increased in the highest level of pH and inoculum size. Therefore it can be concluded that ethanol production increased from 6.2 ± 0.8 g/L to 9.5 ± 1.1 g/L after optimization.

Keywords: Bioethanol, Cocoa waste, Optimization, Response Surface Method, Lignocellulosic

1 Introduction

Since a few decades ago, energy crops and lignocellulosic biomass become the good representatives of the global potential of bio-energy resources. For second-generation fuels, many countries try to produce bioenergy from biomass at a lower cost (Saggay and Dey, 2016; Zayed et al, 2016). The huge availability of woody biomass made it a good candidate for bioenergy production (Ullah et al, 2015). Furthermore one of the important choices for energy production (biofuel) is the conversion of lignocellulosic biomass (Srivasta et al, 2015; Rezanian et al, 2020a). As reported by Morales et al. (2015), usage of agricultural residues can reduce 82% to 91% of greenhouse gas emission in comparison to non-fossil sources of energy production. Agricultural wastes for bioethanol production are more feasible as they are not related to the food supply (Budzianowski, 2012). More attention is paid to the development of methods to produce ethanol from biomass that contains higher carbohydrate content (Idi and Mohamad, 2011; Rezanian et al, 2017; Rezanian et al, 2019). Carbohydrates part of biomass (cellulose and hemicellulose), can be easily converted to the pentose and hexose sugars and the sugars can be converted to bioethanol using fermentation by yeast (Comelli et al, 2016). Based on the previous study, cocoa waste consisted of 10% cellulose, 41% hemicelluloses, and 31% lignin of biomass dry weight (Idi et al, 2012). To enhance the ethanol production rate from lignocellulosic biomass, pretreatment is defined as a required step (Zhou et al, 2014; Kumar et al, 2016). Pretreatment has a significant effect on the composition of biomass in comparison to the untreated biomass (Karimi and Taherzadeh, 2016; Rezanian et al, 2020b).

In Malaysia and Indonesia, tapai is a traditional fermented food. It contains some types of microorganisms that can

ferment sugars to ethanol such as (*Rhizopusoryzae*, *Amylomyces rouxii*, *Mucor sp.* and *Candida utilis*) and yeasts (*Saccharomyces cerevisiae*, *Saccharomycopsis fibuliger*, *Endomycopsis burtonii*) (Gandjar et al, 2003). The traditional method of optimization of parameters involves optimizing one parameter at a time. But the influence of different parameters can be determined experimentally in the laboratory scale (Al-Shorgani et al, 2015; Cavalaglio et al, 2016). Response Surface Methodology (RSM) is a collection of statistical and mathematical techniques that can reduce the number of experimental trials and evaluate the multiple parameters and their interactions (Silva et al, 2011; Singh and Bishnoi, 2013; Wang et al, 2015). It also helps to determine the optimum conditions of the region of the factor while satisfaction of involved parameters is considered (Sampaio et al, 2016). Hence, there is no research for ethanol production from lignocellulosic material like cocoa waste, the main objectives of this study are as follows: (a) optimization of ethanol production from cocoa waste by locally isolated microorganisms using RSM; (b) investigation of interaction between independent variables such as pH, temperature, CW concentration, and inoculum size; and (c) evaluation of the optimized condition based on significant effecting factors and to compare with the results before optimization.

2 Materials and methods

2.1 Microorganism cultivation and preparation

Tapai ubi (TU) and tapai pulut (TP) were bought from a local market in Johor, Malaysia. Then, 10 g of TU and TP were mixed with 100 ml of sterile distilled water and 0.9 g NaCl using a blender (Home-HM3258). To culture, the microorganism, 0.1 ml of the mixture was transferred on the

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Potato Yeast Starter (PYS) medium. Then, incubated at 30°C for 24 hours. Two different types of colonies appeared on the plate of PYS. Yeasts were isolated based on colony color and shape. Finally, the plates transferred to the fridge for further usage (Pérez-Sariñana et al, 2015). From each plate of PYS, a loopful of microorganisms was aseptically transferred into a sterile 250 ml Erlenmeyer containing 100 ml of nutrient broth. The mixture was incubated in a shaker with 150 rpm at 30 °C for 24 hours. Optical density was measured at 600 nm to reach to 0.8 optical density before using microorganisms in all the experiments. Then, 1 mL of samples were taken every 3 h and centrifuged at 10,000 rpm for 10 min. The supernatant was filtered using a 0.22 µm filter (Millipore, Bedford, MA, USA) and stored at -20 °C for further analysis (Pérez-Sariñana et al., 2015). To observe the morphology, a small piece of tape was placed briefly on the surface of the mature colony. Then, a drop of methylene blue was dropped on a piece of the microscope slide. The tape was placed on the microscopic slide observed in 10X to 40X zoom using AmScope B490B Binocular Microscope (made in the USA).

2.2 Pretreatment of cocoa waste

Cocoa waste was ground using a blender to prepare a fine powder. Then, 10 g of biomass powder was mixed with 100 mL of 0.5 M sulfuric acid. The solution was autoclaved for 5 minutes at 121°C and dried in an oven at 70 °C for one day. Then acid hydrolyzed biomass was filtered and further used in fermentation.

2.3 Optimization by response surface method (RSM)

A central composite design (CCD) was performed to find the optimum conditions to maximize ethanol production using (Design-Expert version 9.4, Minneapolis, MN, USA). The CCD has constructed 40 factorial experiments with 2 replications and 6 center points. In addition, it used to evaluate the interactions and effect of four factors including pH, temperature, inoculum size, and CW concentration (Granados-Arvizu et al., 2017).

2.4 Fermentation

Fermentation was performed in 250 ml flasks containing fermentation medium with the following composition in g/L (yeast extract, 5; MgSO₄·peptone, 5; KH₂PO₄, 1; 7H₂O, 0.3; NH₄Cl, 2). Then, pretreated CW and inoculum (microorganisms isolated from TU and TP) added. The fermentation was carried out at pH 7 at 37 °C with shaking at

100 rpm for 60 h in 250 mL Erlenmeyer flasks. Samples were taken every 6 hours and all the experiments were conducted in triplicate.

2.5 Analytical procedures

The composition of CW (cellulose, hemicellulose and lignin) measured according to Di Blasi et al. (1999). Dinitrosalicylic method was used to estimate the total reducing sugar in the enzymatic hydrolysate of acid pretreated CW (Miller, 1959). Ethanol concentration was measured by using Agilent Technologies 6890N gas chromatography equipped with a flame ionization detector (FID) using a non-polar capillary column (0.32mm). The carrier gas was helium and temperature held at 40°C for 4 minutes. A setting of temperature was adjusted from 10°C to 100°C and the detector and injector temperature was set at 250°C. Then, the samples were centrifuged at 3000 rpm for 30 minutes. The pellet discarded and the supernatant was analyzed for ethanol concentration. The ethanol yield was calculated based on Ang et al, (2015):

$$EY (\%) = \frac{\text{ethanol (g/L)}}{(\text{glucose (g/L)} 0.514) + (\text{xylose (g/L)} 0.511)} \times 100$$

where EY is ethanol yield.

3 Results and discussion

Figure 1 shows the morphology of different types of isolated microorganisms. The differences between isolated microorganisms from *tapai ubi* and *tapai pulut* were identified by observation of their growth pattern, size and color of colonies on the agar plate as well as microscopic. In addition, *tapai pulut* colonies were in a spherical shape and yellowish color meanwhile *tapai ubi* were in ellipsoid shape and white color.

3.1 CCD optimization for ethanol production

A 2⁴ full factorial design with three levels leading was performed to determine the most suitable level for the selected variables. The experimental range and levels of independent variables for ethanol production are shown in Table 1. Temperature, pH, inoculum and CW concentration were chosen as independent variables while ethanol production was the dependent variable. Based on Table 2, the range of parameters was: pH (4–6), temperature (25–35°C), CW concentration (0.1–0.3%) and inoculum size (7–11%).

Figure 1: Morphology of isolated microorganisms under light microscope (a) *Tapai ubi* and (b) *Tapai pulut*

Table 1: levels of independent variables in experimental design

Indications	Independent variables	Unit	Low Level	High Level	Mean
A	pH	-	4	6	5
B	Temperature	°C	25	35	30
C	Inoculum	%	7	11	9
D	CW concentration	g/L	0.1	0.3	0.2

As shown in Table 2, total 46 experiments were generated by the software and the response values ranged between 0 to 0.743.

Table 2: Experimental design

Run number	pH	Temperature (°C)	Inoculum (%)	CW Con (%)	Response (ethanol)
1	4.00	25.00	7.00	0.10	0.100
2	4.00	25.00	7.00	0.10	0.128
3	6.00	25.00	7.00	0.10	0.074
4	6.00	25.00	7.00	0.10	0.070
5	4.00	35.00	7.00	0.10	0.260
6	4.00	35.00	7.00	0.10	0.074
7	6.00	35.00	7.00	0.10	0.083
8	6.00	35.00	7.00	0.10	0.052
9	4.00	25.00	11.00	0.10	0.064
10	4.00	25.00	11.00	0.10	0.072
11	6.00	25.00	11.00	0.10	0.064
12	6.00	25.00	11.00	0.10	0.063
13	4.00	35.00	11.00	0.10	0.041
14	4.00	35.00	11.00	0.10	0.000
15	6.00	35.00	11.00	0.10	0.053
16	6.00	35.00	11.00	0.10	0.000
17	4.00	25.00	7.00	0.30	0.063
18	4.00	25.00	7.00	0.30	0.062
19	6.00	25.00	7.00	0.30	0.063
20	6.00	25.00	7.00	0.30	0.065
21	4.00	35.00	7.00	0.30	0.037
22	4.00	35.00	7.00	0.30	0.040
23	6.00	35.00	7.00	0.30	0.070
24	6.00	35.00	7.00	0.30	0.070
25	4.00	25.00	11.00	0.30	0.065
26	4.00	25.00	11.00	0.30	0.054
27	6.00	25.00	11.00	0.30	0.053
28	6.00	25.00	11.00	0.30	0.064
29	4.00	35.00	11.00	0.30	0.122
30	4.00	35.00	11.00	0.30	0.099
31	6.00	35.00	11.00	0.30	0.094
32	6.00	35.00	11.00	0.30	0.116
33	2.62	30.00	9.00	0.20	0.047
34	7.38	30.00	9.00	0.20	0.743
35	5.00	18.11	9.00	0.20	0.000
36	5.00	41.89	9.00	0.20	0.010
37	5.00	30.00	4.24	0.20	0.000
38	5.00	30.00	13.76	0.20	0.000
39	5.00	30.00	9.00	0.04	0.096
40	5.00	30.00	9.00	0.44	0.608
41	5.00	30.00	9.00	0.20	0.000
42	5.00	30.00	9.00	0.20	0.115
43	5.00	30.00	9.00	0.20	0.262
44	5.00	30.00	9.00	0.20	0.000
45	5.00	30.00	9.00	0.20	0.000
46	5.00	30.00	9.00	0.20	0.000

3.2 ANOVA Analysis

ANOVA analysis was used to evaluate the effect of pH (factor A), temperature (factor B), inoculum size (factor C) and the CW concentration (factor D) as independent variables of the fermentation, and ethanol concentration (Y) in g/L. The following equation (Eq. 1) was used to build surface graphs for the model for each response variable and for predicting the optimal value as follows:

$$Y(\text{g/L}) = 0.027 + 0.033 A + 2.558E-003 B - 6.626E-003 C + 0.027 D - 1.344E-003 AB + 6.469E-003 AC + 0.010 AD - 1.094E-003 BC + 7.219E-003 BD + 0.021 CD + 0.054 A^2 - 0.015 B^2 - 0.016 C^2 + 0.046 D^2$$

Table 3 shows the summary of ANOVA analysis. The fit of the model was checked with the coefficient of determination R^2 , which was calculated 0.976. This indicates that the model can be considered statistically significant with 95% confidence, with F-value of 2.04 while ' $P > F$ ' (0.0487) and the model was significant. The probability p-value was lower than (0.05), indicating the significance of the model according to ANOVA which implies model adequacy. The final equation in terms of actual values is shown as follows:

3.3 Interaction of influenced factors for bioethanol production

There are different parameters that have an effect on ethanol production from lignocellulosic materials such as CW. The interaction effects of the independent variables for ethanol production were analyzed using RSM. It should be noted that there is no synergetic effect of multiple inhibitory compounds in the fermentations containing a variety of toxic compounds (Wyman, 2018). Three-dimensional response surface and contour plots were generated to evaluate the interactions between two variables. This was performed by evaluating two variables at a time while fixing the remaining variable at a central level which is shown in Figures 2 to 4.

3.3.1 Interaction of temperature with CW concentration and inoculum (effect of pretreatment temperature and retention time)

The effect of temperature and cocoa waste concentration on ethanol production is shown in Figure 2A. base on the results, ethanol production increased proportionally with an increase in temperature from 27.5 °C to 32.5°C. Hence, CW concentration increased while the ethanol concentration was constant. Figure 2B shows that ethanol production increased significantly while the inoculum was in the middle range (9%). Similarly, by increasing the temperature, a smooth decline in ethanol production observed. Subsequently, these two parameters had the highest contribution in ethanol production.

Table 3: ANOVA for Response Surface Quadratic Model

Source	Sum of Squares	Degree of freedom	Mean Squares	F value	P> F	R2
Model	0.41	14	0.029	2.04	0.0487	significant
Residual	0.45	31	0.014	-	-	-
Lack of fit	0.37	10	0.037	9.76	>0.0001	-
Pure error	0.079	21	3.755E003	-	-	-
Total	0.86	45	-	-	-	-

F-value is significant.

Model is significant, with $P > F$ less than 0.05

Figure 2: (A) Response surface plots of the effects of temperature and CW concentration and (B) Response surface plots of the effects of temperature and inoculum

3.3.2 Interaction of pH with temperature and inoculum

Figure 3A shows the interaction between inoculum and pH on ethanol production. Based on the results, by increasing the inoculum size, ethanol production was increased. Moreover, in the higher pH, the microorganism had more activity that resulted in higher ethanol production rate. In comparison to Figure 3B, ethanol production was increased in the high level of pH and constant level of temperature. As mentioned earlier, ethanol production increased at the middle range of inoculum and temperature. Consequently, higher ethanol concentration obtained in higher pH and constant rate of the inoculum size. Interaction of pH and temperature showed that they have the same effects on ethanol production. It means by increasing pH and temperature the ethanol production was increased. Based on Figure 3, pH was the most significant variable for ethanol production. It indicates that in higher pH, the production was a higher while, the temperature was not contributed significantly

in ethanol production. The interactive 3D plots in Figure 4 showed that ethanol production had affected by increasing the inoculum size and pH. Hence, as CW concentration increased, a higher amount of ethanol was produced in a high level of pH. As a result, these two factors directly had direct effects on ethanol production.

3.3.3 Interaction of CW concentration with pH and inoculum

Figure 4A and 4B shows the interaction between CW concentration with inoculum and pH which had significant effects on ethanol production. Figure 4A shows that ethanol production increased when the CW concentration and pH were at the highest level. In contrast, as the inoculum size increased, a smooth decrease in ethanol production observed. As the CW concentration increased, the ethanol production increased constantly which was 0.3 g/L at the highest level (Figure 4B).

Figure 3: (A) Response surface plots of the effects of pH and inoculum and (B) Response surface plots of the effects of pH and temperature

Figure 4: (A) Response surface plots of the effects of CW concentration and pH and (B) Response surface plots of the effects of CW concentration and inoculum

Table 4: Optimum values of the process parameter for maximum efficiency

Parameters	Before optimization	After optimization
	Suboptimum conditions	Optimum values
pH	5	6
Temperature (°C)	30	31.5
Inoculum size (%)	9	10.5
Cocoa waste concentration (g/L)	0.2	0.3
Rate of bioethanol production (g/L)	6.2 ± 0.8	9.5 ± 1.1

Inoculum size directly influenced the ethanol production as there was a high production rate at all the ratio of inoculum. By combining the results in Figures 3 and 4, it can be concluded that the highest ethanol production by 0.3 mg/L obtained in the higher level of pH (5 to 6) (Figure 3B). In the highest range of CW, ethanol production was low while the temperature and CW concentration were decreased. It showed a positive effect of CW concentration and pH in the quadratic model.

3.4 Response of optimization

The optimal conditions derived from the quadratic models were pH 6; temperature 31.5°C; inoculum size 10.5% and CW concentration 0.3 g/L. In the experimental results, the highest ethanol production was 9.5 ± 1.1 g/L which is higher than the suboptimum condition 6.2 ± 0.8 g/L. As reported by Idi et al, (2012), 7.911 g/L of ethanol was produced using acid pretreated CW without optimization. In several studies, other lignocellulosic also have been used for ethanol production using RSM. For instance, 4.28 g/L ethanol obtained from acid pretreated sugarcane bagasse in the optimized condition of temperature 33°C, pH 5.7, and fermentation time of 104 h (Ezhumalai and Thangavelu, 2010). Table 4 shows the ethanol production rate during the operation of the bioreactor in optimum conditions was approximately 50% higher than the operation of same the bioreactor in suboptimum conditions.

5 Conclusion

In this study ethanol production from cocoa waste by using locally isolated microorganisms were optimized using RSM. The interaction of influenced parameters such as temperature, CW concentration, inoculum size, and pH was investigated. Based on RSM, total of 46 experiments generated to obtain the optimized condition. The isolated microorganisms from both *tapai pulut* and *tapai ubi* were able to produce ethanol. The results proved that all the parameters had a contribution in ethanol production but the most dominant factors were pH and inoculum size. The optimized condition was at pH 6, temperature 31.5 °C, inoculum size 10.5%, and CW concentration of 0.3 g/L. The highest ethanol production was 9.5 ± 1.1 g/L using RSM and 6.2 ± 0.8 g/L before optimization. Further studies in a different condition of fermentation and using different microorganisms should be conducted to enhance ethanol production from CW.

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