Research Article
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Bioethanol production from cocoa waste by locally isolated microorganism using response surface methodology

Abstract

The rate of ethanol production can be affected by different parameters that involved during fermentation. In this study, acid treated cocoa waste (CW) was used as a lignocellulosic substrate for ethanol production in the simultaneous saccharification and fermentation (SSF) using microorganism isolated from locally fermented food tapai ubi and tapai pulut. For optimization, the experiments were carried out using response surface methodology (RSM). The effect of four independent variables temperature, CW concentration, inoculum size and pH during fermentation was investigated. A central composite design (CCD) was used to evaluate the effect and interactions of the parameters. ANOVA analysis revealed that pH and inoculum size had the most significant effects on the ethanol production. The optimized condition for the ethanol production was at temperature 31.7°C, pH 6.0, inoculum size 10.5% and CW concentration 0.3g/L while after optimization, ethanol podcution increased from 6.2±0.8g/L to 9.5±1.1g/L.

Keywords: bioethanol, cocoa waste, optimization, response surface method, lignocellulosic

Volume 3 Issue 4 - 2018

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Received: March 16, 2018 | **Published:** September 18, 2018

Introduction

In the last three decades, the statistics shows the rate of CO_2 generation has increased sharply.[1,2] Energy crops and lignocellulosic biomass are good representative of global potential of bio-energy resources. For second generation fuels, many countries tries to produce bioenergy from biomass at the lower cost. Huge availability of woody biomass made it as a good candidate for bioenergy production.[3] Furthermore one of the important choices for energy production (biofuel) is the conversion of lignocellulosic biomass.[4,5]

Agricultural wastes are a suitable substrate for bioethanol production which can be replaced by fossil fuels as they are cheap and eco-friendly.[6] As reported by Morales et al.,[7] usage of agricultural residues can reduce 82% to 91% of greenhouse has emission (GHG) in compare to non fossil sources of energy production. Agricultural product waste for bioethanol production are more feasible as they are not related to food supply.[8] Much attaention is channeled to the development of methods to produce ethanol from biomass that contains higher carbohydrate content.[9–11] 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.[12] Based of the previous study, cocoa waste consisted of 10% celloluse, 41% hemicelluloses and 31% lignin of biomass dry weight.[13] To enhance the ethanol production rate from lignocellulosic biomass, pretreatment is defined as a required step.[14,15] Pretreatment has significant effect on the composition of biomass in compare to the untreated biomass.[16]

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*).[17] The traditional method of optimization of parameters involves optimizing one parameter at a time. But the influence of different parameters can be determined experimentally on a laboratory scale.[18,19] Response Surface Methodology (RSM) is a collection of statistical and mathematical techniques which can reduce the number of experimental trials and evaluate the multiple parameters and their interactions.[20–22] It also helps to determine the optimum conditions of region of the factor while a satisfaction of involved parameters is considered.[23]

Hence, there is no research for ethanol production from lignicellulosic material like cocoa waste; the main objectives of this study are as follows:

a. Optimization of the ethanol production from cocoa waste by locally isolated microorganism suing RSM.

b. Investigation of interaction between independent variables such as pH, temperature, CW concentration and inoculum size.

c. Evaluation of the optimized condition based on significant effecting factors and to compare with the results before optimization.

Materials and methods

Isolation, culture and screening of microorganism from *tapai ubi* and *tapai pulut*

Tapai ubi (TU) and *tapai pulut* (TP) were bought from a local market in Johor, Malaysia. Then, 10g of TU and TP was mixed with 100ml of sterile distilled water and 0.9g NaCl using a blender (Home-HM3258). To culture the microorganism, 0.1ml of the mixture was transferred on the Potato Yeast Starter (PYS) medium. Then incubated at 30°C for 24hours.[24] 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.

From each plate of PYS, a loopful of microorganisms was aseptically transferred in a sterile 250ml Erlenmeyer containing 100ml of nutrient broth and incubated in a shaker incubator (150rpm) at 30°C for 24 hours. Optical density (OD) at 600nm was measured to reach to 0.8 optical density (satisfactory level) before using microorganisms in all the experiments. To observe the morphology, a small pierce of tape was placed briefly on the surface of the mature colony. Then, a drop of methylene blue was dropped on a pierce of microscope slide. The tape was placed on the microscopic slide with 40X zoom and observed using Am Scope B490B Binocular Microscope.

Pretreatment of cocoa waste

Cocoa waste was grinded using blender to prepare a fine powder. Then, 50g of biomass powder was mixed with 500mL 0.5M sulfuric acid. The solution was autoclaved for 5minutes at 121°C and dried in oven at 70°C for one day. Then acid hydroslated biomass was filtered and further used in fermentation.

Optimization by response surface method (RSM)

In this study, central composite design (CCD) was used to evaluate the interactions and effect of four factors including pH, temperature, inoculum and cocoa waste concentration for ethanol production. The experiments were designed and analyzed using Design Expert Software version 6.4 and each expriement done in triplicate.

Fermentation

Fermentation was performed in 250 ml flasks containing fermentation medium with the following composition in g/L (yeast extract, 5; $MgSO_4$·peptone, 5; KH_2PO_4, 1; $7H_2O$, 0.3; NH_4Cl, 2). Then, pretreated CW and inoculum (microorganisms isolated from TU and TP) added. The fermentation was carried out at pH) in 37°C with shaking at 100 rpm for 60h in 250mL erlenmeyer flasks. Samples were taken every 6hours and all the expriements were conducted in triplicate.

Ethanol detemination by gas chromatography

Ethanol concentration was determined by using Agilent Technologies 6890N gas chromatography equipped with flame ionization detector (FID) using a non-polar capillary column (0.32mm). The carrier gas was helium and temperature held at 40°C for 4minutes. Setting of temperature was adjusted with increment of temperature from 10°C to 100°C and detector and injector temperature were set at 250°C. Then, the sapmles were centrifuged at 3000rpm for 30minutes. The pallet discarded and supernatant contained sample in dichloromethane was taken out for ethanol analysis. The ethanol yield was calculated based on Ang et al.[25]

$$Ethanol\ yield\ (\%) = \frac{ethanol\,(g/L)}{(glucose\,(g/L)\,0.514)(xylose\,(g/L)\,0.511)} \times 100$$

Results and discussion

Figure 1 shows the morphology of different types of isolated microorganisms. The differences between isolated microorganism 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 spherical shape and yellowish color meanwhile *tapai ubi* colonies were with color with ellipsoid shape. Then, isolated microorganisms were used to ferment the pretreated CW.

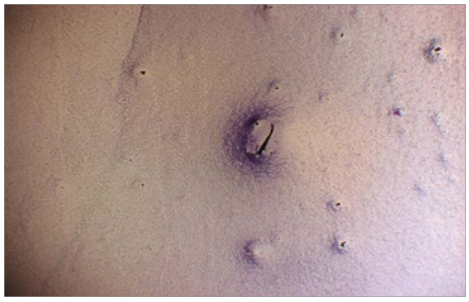

Figure 1(A) Morphology of isolated microorganisms under light microscope *Tapai ubi.*

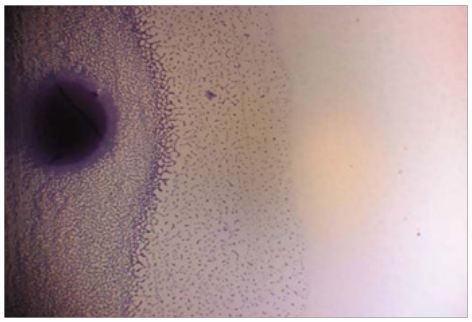

Figure 1(B) Morphology of isolated microorganisms under light microscope *Tapai pulut.*

CCD optimization of ethanol production

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 ranges of parameters were: pH (4–6), temperature (25–35°C), CW concentration (0.1–0.3%) and inoculum size (7–11%).

Table 1 level of independent variables in experimental design

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

Response surface methodology (RSM) with full fractionate central composite design (CCD) was performed to determine the most suitable level for the selected variables. As shown in Table 2, totally 46 experiments was generated by the software that including 40 factorial experiments with 2 replications and 6 center points.

ANOVA analysis

Table 3 shows the summary of ANOVA analysis. The fit of the model was checked with the co efficient of determination R^2, which was calculated 0.976. This indicates that the model can be considered

statistically significant with 95% of 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 on model adequacy. The final equation in terms of actual values is shown as follows:

$$Y(g/L) = 0.027 + 0.033\,A + 2.558E-003\,B - 6.626E-003\,C + 0.027\,D - 1.344E-003\,AB + 6.469E-003\,A\,C + 0.010$$

$$AD - 1.094E-003\,BC + 7.219E-003\,BD + 0.021\,CD + 0.054\,A^2 - 0.015\,B^2 - 0.016\,C^2 + 0.046D^2$$

Where Y represents ethanol concentration (g/L) while A, B, C, and D represents pH, temperature, inoculum and CW concentration, respectively. This regression model was generated by design expert software, after considering all the variables.

Table 2 Experimental design

Run number	pH	Temperature (°C)	Inoculum (%)	CW Con (%)	Response
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

Table continued...

Run number	pH	Temperature (°C)	Inoculum (%)	CW Con (%)	Response
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

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	-	-	-	-

FL-value is significant; Model is significant, with P>F less than 0.05

Table 4 Optimum values of the process parameter for maximum efficiency

	Before optimization	After optimization
Parameters	**Suboptimum conditions**	**Optimum values**
pH	5	6
Temperature (°C)	30	31.5
Inoculum size (%)	9	10.5

Citation: Rezania R, Mohamad SE, Yahya A, et al. Bioethanol production from cocoa waste by locally isolated microorganism using response surface methodology. *MOJ Biol Med.* 2018;3(4):160–166. DOI: 10.15406/mojbm.2018.03.00092

Table continued...

	Before optimization	After optimization
Parameters	**Suboptimum conditions**	**Optimum values**
Cocoa waste concentration (g/L)	0.2	0.3
Rate of bioethanol production (g/L)	6.2±0.8	9.5±1.1

Interaction of influenced factors for bioethanol production

The response surface 3D plots, which are the graphical results of interactive effects, are shown in Figures 2–4.

Interaction of temperature with CW concentration and inoculum

The effect of temperature and cocoa waste concentration on ethanol production are 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 indicates that ethanol production in the middle range of inoculum (9%) increased significantly. Similarly, by increasing the temperature, a smooth decline in ethanol production observed. Subsequently, these two parameters had highest contribution in ethanol production.

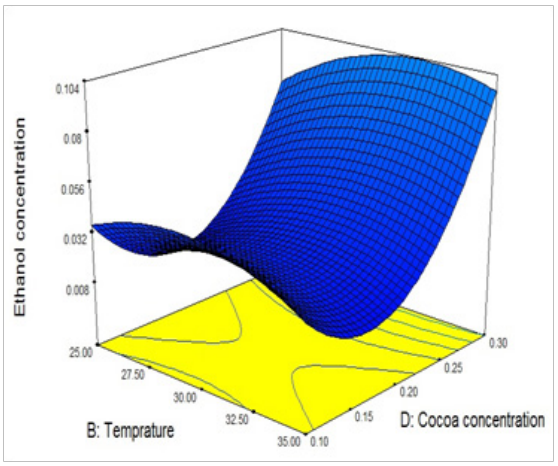

Figure 2(A) Effects of temperature and CW concentration.

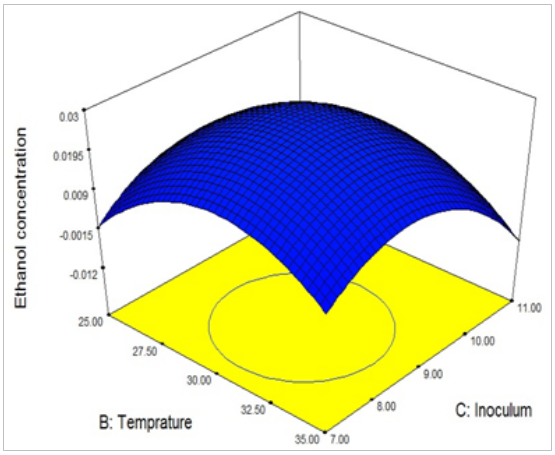

Figure 2(B) Effects of temperature and inoculum.

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 in inoculum size, ethanol concentration increased. In higher pH, the microoganism had more activity that resulted in higher ethanol production rate. In comparison to Figure 3B, ethanol production was increased in high level of pH and constant level of temprature,.

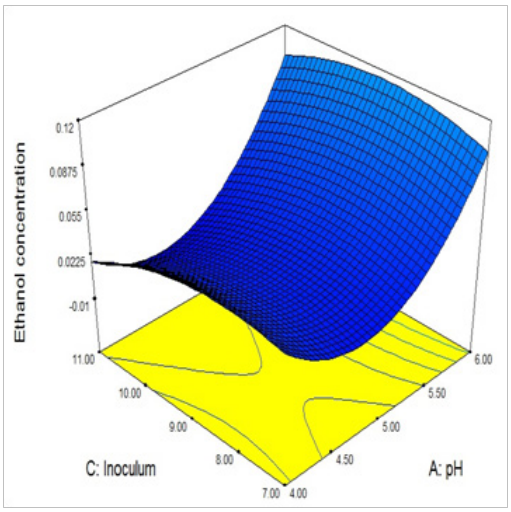

Figure 3(A) Effects of pH and inoculum.

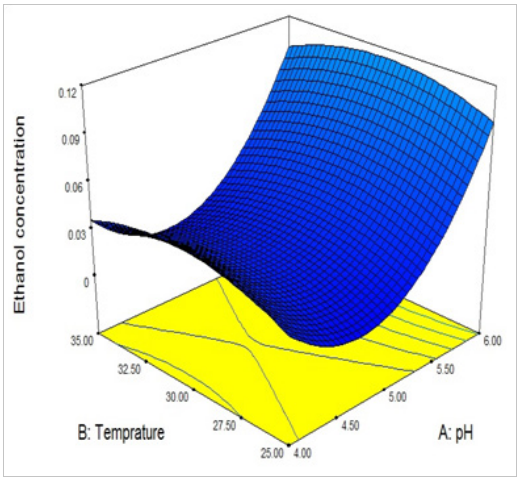

Figure 3(B) Effects of pH and temperature.

As mentioned earlier, ethanol production increased at middle range of inoculum and temperature. Consequently, higher ethanol concentration obtained while pH increased and the inoculum size was constant. Interaction of pH and temperature showed that they have same effect on ethanol production. It means by increasing pH and temperature the athanol production increased. The 3D response surface plots in Figure 3 shows pH was the most significant variable

for ethanol production. It indicates that in higher pH, the production was higher while, temperature was not contributed significntly in ethanol production.

The interactive 3D plots in Figure 4 showed that ethanol production had affected by increasing the inoculum size and pH. By increment of the CW concentration, higher amount of ethanol was produced in high level of pH. These two factors directly had direct effects on ethanol production.

Interaction of CW concentration with pH and inoculum

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

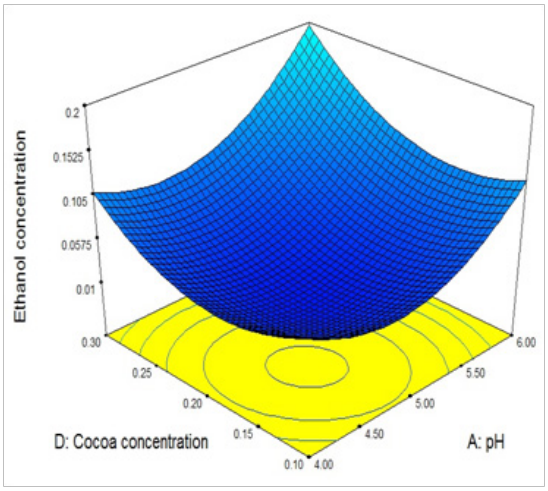

Figure 4(A) Effects of CW concentration and pH.

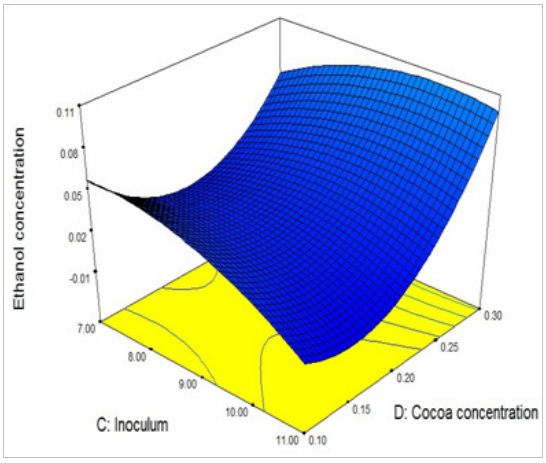

Figure 4(B) Effects of CW concentration and inoculum.

Inoculum size influenced the ethanol production directly which showed highest production rate at the the all ratio of inoculum. By combining the results in Figures 3&4, it can be cocluded that the highest ethanol production by 0.3mg/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 decreased. It showed a positive effects of CW concentration and pH in quadratic model.

Optimum condition

As shown in Figures 2–4 the design expert was useful to optimize the ethanol production condition. The optimum conditions was as follows: pH 6; temperature 31.5°C; inoculum size 10.5% and CW concentration 0.3g/L. In optimum condition, ethanol production was 9.5±1.1g/L which is higher than suboptimum condition at 6.2±0.8g/L. As reported earlier by Idi et al.,[13] 7.911g/L of ethanol is produced using sulfuric acid pretreated cocoa waste. Table 4 shows the ethanol production rate during operation of bioreactor in optimum conditions was approximately 50% higher than operation of same bioreactor in suboptimum conditions.

Conclusion

In this study ethanol production from cocoa waste by using locally isolated microorganism was optimized by RSM. The interaction of influenced parameters such as temperature, CW concentration, inoculum size and pH was investigated. Based on RSM, totally 46 expriments 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 contribution in ethanol production but the most dominant factors were pH and inoculum size. The optimaized condition was at pH 6, temperature 31.5°C, inoculum size 10.5% and CW concentration of 0.3g/L. Highest ethanol production was at 9.5±1.1g/L using RSM and 6.2±0.8g/L before optimization.

Acknowledgments

The authors of this paper would like to show their deepest gratitude to Faculty of Biosciences and Bioengineering (UTM) for providing us the lab space and support to carry out this research.

Conflict of interest

All the authors declare that they have no conflict of interest.

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Citation: Rezania R, Mohamad SE, Yahya A, et al. Bioethanol production from cocoa waste by locally isolated microorganism using response surface methodology. *MOJ Biol Med.* 2018;3(4):160–166. DOI: 10.15406/mojbm.2018.03.00092