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Full Length Research Paper

Production of sugar by hydrolysis of empty fruit bunches using palm oil mill effluent (POME) based cellulases: Optimization study

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The utilization of lignocellulosic materials such as empty fruit bunches (EFB) from palm oil plant for bioethanol production attract increasing attention as an abundantly available and cheap renewable residue, especially in Malaysia where palm oil production is the major agricultural industry. The most challenging part in conversion of lignocellulosic materials to bioethanol is the hydrolysis process in order to obtain a reducing sugar. In this study, cellulase enzyme used for the hydrolysis was produced from palm oil mill effluent (POME), whose cost of production was considerably low as compared to commercial cellulases. The hydrolysis of EFB for sugar production as an initial step was statistically optimized based on agitation speed, EFB and cellulase concentrations using response surface methodology (RSM) through Box-Behnken design in 2 L bioreactor. The reducing sugar obtained is 16.85 g/L, which appeared at substrate concentration of 5.91%, enzyme concentration of 4.88% and agitation of 233 rpm.

Key words: Reducing sugar, empty fruit bunches (EFB), Box-Benhken design, lignocellulosic, hydrolysis, cellulose.

INTRODUCTION

Alternative energy resources have been the focus nowadays in view of continuously rising petroleum costs in the world. One of renewable energy resource that received major attention is ethanol (Demirbas, 2005). The production of ethanol using crops and sugar cane as raw material is well-established and there are many concerns related to this issue regarding the use of food based as starting raw material for ethanol production (Sukumaran et al., 2009). It is believed that lignocellulosic material has the potential for conversion into sugars and ethanol but the process is quite complex as compared to crop based techniques (Zaldivar et al., 2001). Lignocelluloses are the most abundant materials that are generated through agricultural practices and their component include cellulose, hemicellulose and lignin (Lin and Tanaka, 2006). Cellulose molecules consist of long chains of glucose units, while hemicelluloses comprised pentose molecules (Demirbas, 2005). An example of lignocellulosic material that is abundant in Malaysia is empty fruit bunches (EFB) from palm oil tree. The EFB is the residual bunch after removal of the fruits and it constitutes 23% of the weight of the fresh fruit bunches. The high content of cellulose and hemicelluloses which is about 75 to 80% opens new approaches to convert EFB into bioethanol (Tan et al., 2010).

There are three major steps to be employed in the conversion of lignocellulosic to bioethanol which are pretreatment for lignin breakdown, hydrolysis and fermentation for bioethanol. The most challenging part is the hydrolysis process in order to obtain the reducing sugar. Hydrolysis of lignocellulosic can be done in two ways, either by using enzymatic or chemical methods. However,

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Abbreviations: EFB, Empty fruit bunches; **POME,** palm oil mill effluent; **RSM,** response surface methodology.

Table 1. Experimental and predicted amount of reducing sugar produced.

Run	Substrate concentration (%)	Enzyme concentration (%)	Agitation (rpm)	Reducing sugar produced (g/l)	
				Actual	Predicted
1	4	2	300	11.25	11.12
2	7	5	300	14.24	14.23
3	4	8	200	13.21	13.35
4	7	8	250	15.95	15.84
5	4	5	250	16.23	16.34
6	7	5	200	14.32	14.29
7	4	5	250	16.38	16.34
8	1	5	200	3.93	3.94
9	4	5	250	15.98	16.34
10	4	2	200	10.35	10.23
11	4	8	300	12.15	12.27
12	1	5	300	3.78	3.81
13	7	2	250	12.43	12.57
14	4	5	250	16.75	16.34
15	4	5	250	16.34	16.34
16	1	2	250	3.21	3.32
17	1	8	250	4.46	4.32

enzymatic hydrolysis is more environmental friendly as compared to chemical hydrolysis. Although, the costs of using commercial enzymes are expensive, however this problem can be ameliorated by producing the enzyme (cellulose) locally in the laboratory using renewable substrate with minimal production cost (Sukumaran et al., 2009). The low cost enzyme can be produced from substrate such as palm oil mill effluent (POME), where POME is a thick brownish liquid that contains high amounts of total solids (40,500 mg/L), oil and grease (4000 mg/L), COD (50,000 mg/L) and BOD (25,000 mg/L). The disposal of this highly polluting effluent is becoming a major problem if it is not being treated properly. Therefore, this POME can be used as substrate for cellulase production hence it can reduce the environmental pollution (Abdul Latiff et al., 2003).In this study, three factors that affect reducing sugar production by EFB (agitation, substrate concentration and enzyme concentration) were optimized by response surface methodology using Box-Benhken design. The locally produced cellulase enzyme from *Trichoderma reesei* RUT C30 was used during the hydrolysis process.

MATERIALS AND METHODS

Substrate collection

Shredded EFB was obtained from Sime Darby Plantation Sdn. Bhd., Carey Island, Malaysia. The samples were washed with normal tap water in order to remove any dirt and oil stains. The washed samples were oven-dried at 90°C for three consecutive days and then milled to 1 mm size using the grinder.

Pre-treatment of substrate

Alkali (sodium hydroxide, NaOH) pre-treatment process was used in this study, making the ratio of sample to 3% NaOH to be 1:4. The mixture was incubated for 2 h at 100°C in water bath and washed several times with water to neutralize it. The pre-treated sample was oven-dried at 60°C for 24 h before it could be used.

Enzyme production

Cellulase used for enzymatic hydrolysis of EFB was collected from the laboratory stock produced by *T. reesei* RUT C30 from palm oil mill effluent as a basal medium (Rashid et al., 2009).

Optimization of hydrolysis process

The hydrolysis of EFB for sugar production was statistically optimized based on three factors which are agitation speed, substrate and enzyme concentration using response surface methodology (RSM) by Box-Benhken design. The experimental design used for the study is shown in Table 1, where there are 17 experimental runs with five replicated center points. The independent variables were studied at three different levels: low, medium and high. The temperature, pH and time were fixed at 50°C, pH 4.5 and 3 days. Enzymatic hydrolysis was performed in 2-L bioreactor containing 1 L mixture of pre-treated EFB, sodium acetate buffer of pH 4.5 and cellulase enzyme according to the design. Analysis of reducing sugar was determined every 24 h. Amount of reducing sugar produced (Y_i, g/l) was used as the dependent output variable. The second degree polynomials (1) were calculated with the statistical package to estimate the response of the dependent variable:

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

Table 2. Analysis of variance of quadratic model for reducing sugar production.

Source	Sum of squares	DF	Mean square	F Value	Probability > F
Model	391.1	9	43.46	688.37	< 0.0001
A	215.9	1	215.9	3420.11	< 0.0001
B	9.1	1	9.1	144.07	< 0.0001
C	0.019	1	0.019	0.3	0.6002
A2	105.18	1	105.18	1666.13	< 0.0001
B2	22.77	1	22.77	360.7	< 0.0001
C2	21.71	1	21.71	343.84	< 0.0001
AB	1.29	1	1.29	20.41	0.0027
AC	1.23E-03	1	-	0.019	0.8931
BC	0.96	1	0.96	15.21	0.0059
Residual	0.44	7	0.063	-	-
Lack of fit	0.13	3	0.044	0.56	0.6696

R^2 = 0.9989, adjusted R^2 = 0.9974.

Where, Y_i is the predicted response; X_1, X_2 , X_3 are the independent variables; β_0 is the offset term; β_1, β_2, β_3 are the linear effects; β_{11}, β_{22}, β_{33} are the squared effects and β_{12}, β_{23}, β_{13} are the interaction terms.

Analytical analysis

The reducing sugars released were measured using 3,5-dinitro-salysilic acid (DNSA) (Miller, 1959) while cellulase activity was determined using carboxymethylcellulase (CMC) assay (Ghose, 1987).

RESULTS AND DISCUSSION

The most challenging part in the production of bioethanol from lignocelluloses materials is the hydrolysis in order to obtain reducing sugar. In this experiment, seventeen runs were performed using statistical Box-Benhken and the second order polynomial equation that gives the amount of reducing sugar produced as a function of substrate concentration (X_1), enzyme concentration (X_2) and agitation (X_3). Table 1 shows the experimental together with the predicted reducing sugar produced for each run. The results demonstrate that the highest amount of reducing sugar produced from hydrolysis process of EFB are 15.98 to 16.75 g/l which can be observed in run 5, 7, 9, 14 and 15. These runs represent the center point of this design. The lowest amount of reducing sugar produced is 3.21 g/l which was observed in run 16. A polynomial regression model is developed by considering the linear, quadratic and interactions effects on the response (reducing sugar) and is presented in Equation 2:

$$\text{Amount of reducing sugar } (Y_j, \text{ g/l}) = 16.34 + 5.19X_1 + 1.07X_2 - 0.049X_3 - 5.00X_1^2 - 2.33X_2^2 - 2.27X_3^2 + 0.57X_1X_2 + 0.017X_1X_3 - 0.49X_2X_3 \quad (2)$$

Analysis of variance (ANOVA) for this experiment is presented in Table 2 where the model of F-value of 688.37 and *p*-value of <0.0001 implies that the model is significant, and there is only a 0.01% chance that a model F value could occur due to noise. Values of $p >$ F less than 0.05 indicate that model terms are significant, while values greater than 0.1 indicate that the model is not significant. The lack of fit value obtained in this experiment is not significant which indicate that the experimental responses sufficiently fit with the model. The R^2 value 0.9989 implies that the sample variation of 99.89% for reducing sugar produced is attributed to the independent variables which are amount of substrate, enzyme concentration and agitation. The R^2 value close to 1 indicates that the model is more fit and denotes a better correlation between the observed and predicted values. Adequate precision is a measure of signal to noise ratio and ratio greater than 4 is desirable. In this experiment, the adequate precision value is 67.551 which indicate an adequate signal and the model can be used to navigate the design space.

The 3D response surface plots for the optimization of hydrolysis process of reducing sugar production is used to investigate the interaction among variables and to determine the optimum concentration of each factor for maximum reducing sugar production from EFB (Tanyildizi et al., 2005). The main target of response surface is to look for efficiently, the optimum values of the variables such that the response is maximized (Tanyildizi et al., 2005). Each contour curve represents an infinite number of combinations of the two test variables, while the other variable was maintained at zero level (centre). The maximum predicted value is identified by the surface confined in the smallest ellipse in the contour diagram. Elliptical contours are obtained when there is a perfect interaction between the independent variables (Muralidhar et al., 2001).

Figure 1 represents the interaction between agitation and enzyme concentration. The reducing sugar produc-

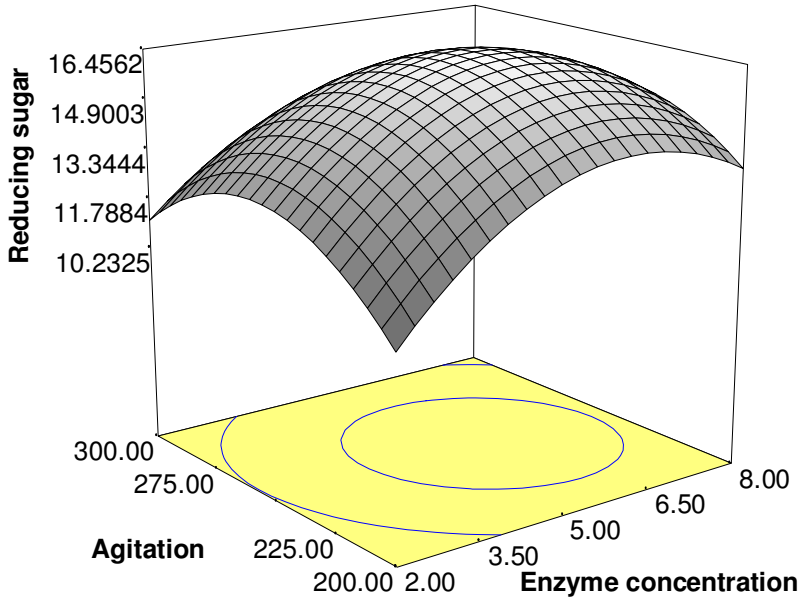

Figure 1. 3D response surface showing the effect of enzyme concentration and agitation on reducing sugar production.

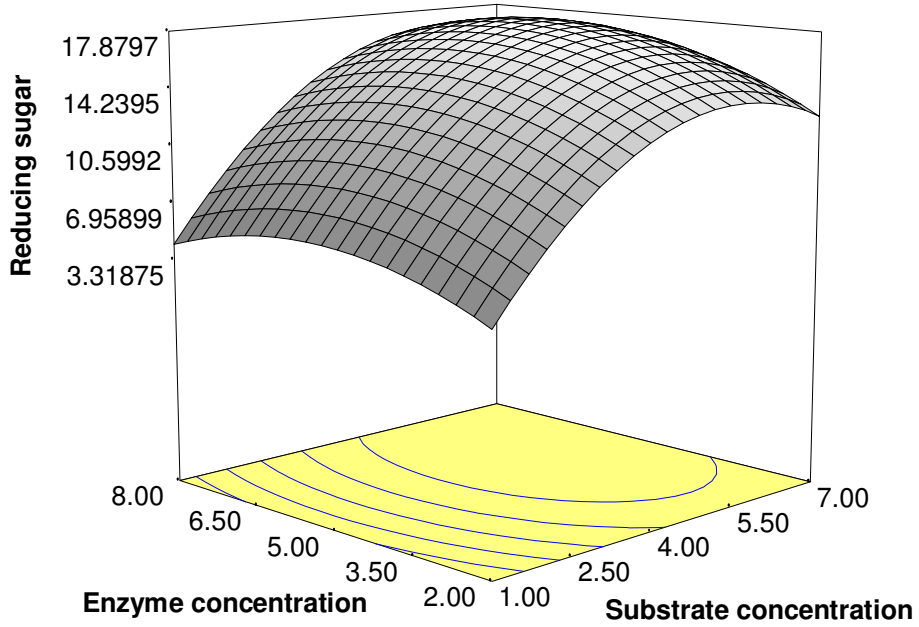

Figure 2. 3D response surface showing the effect of enzyme concentration and substrate concentration on reducing sugar production.

tion decreased at the higher and lower values of ranges for both agitation and enzyme concentration. Maximum production was obtained near the center points of the response surface. Figure 2 represents the interaction between substrate concentration and enzyme concentration, while Figure 3 shows that of agitation and substrate concentration. The shape of the response surfaces curve showed that an increased in substrate concentration will lead to an increase in the reducing sugar production. The maximum reducing sugar production after the validation experiments suggested the optimum parameters based on Design Expert Software to be 16.85 g/L, at substrate, enzyme concentration and agitation of hydrolysis of 5.91%, 4.88% and 233 rpm, respectively.

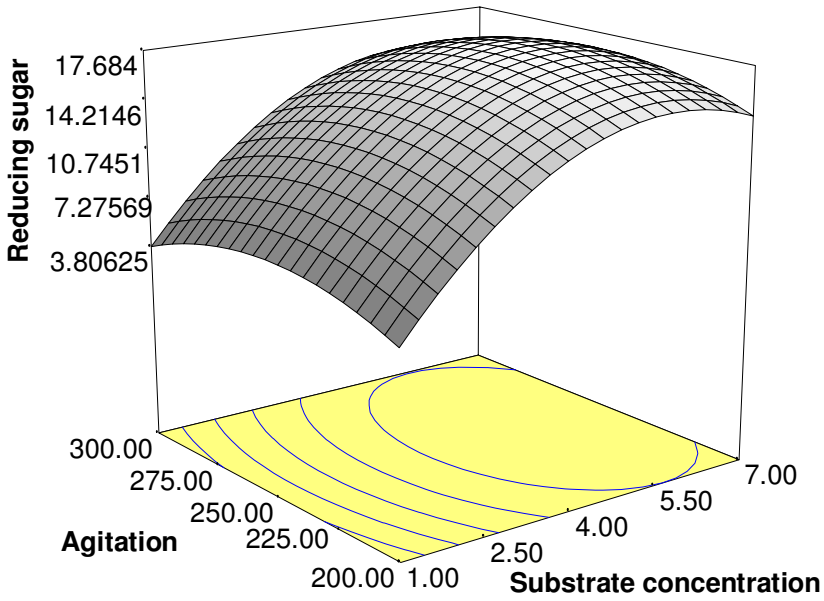

Figure 3. 3D response surface showing the effect of substrate concentration and agitation on reducing sugar production.

There are several literatures regarding reducing sugar production using different lignocellulosic substrate that has been reported. Lignocellulose substrate such as maize straw can produce 89.5 g/L reducing sugar with hydrolysis yield of 83.3% in fed batch hydrolysis. The higher amount of reducing sugar produced is as a result of supplementing cellobiose from *Aspergillus niger* ZU-07, that can greatly reduce the inhibitory effect caused by cellobiose.

The accumulation of cellobiose caused severe feedback inhibition to the cellulase reaction, as the enzyme is more susceptible to end-product-inhibition caused by cellobiose than glucose (Chen et al., 2008). Results also showed that optimum conditions for EFB hydrolysis were obtained with sodium acetate buffer as hydrolysis medium and enzyme dosage of 2:1 (enzyme solution to EFB). Besides that autoclaving the EFB after alkaline pretreatment allows high fiber conversions into reducing sugars (Piarpuzan et al., 2011).

Conclusion

This study shows the reducing sugar production from empty fruit bunches (EFB) by enzymatic hydrolysis using optimization process through Box-Benhken design. The maximum reducing sugar obtained is 16.85 g/L, which appeared at substrate concentration, enzyme concentration and agitation of 5.91%, 4.88% and 233 rpm, respectively.

Therefore, the reducing sugar produced from hydrolysis process can be used in the next step for bioethanol production from EFB.

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