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Statistical Optimization of Fermentation Conditions for Cellulase Production from Palm Oil Mill Effluent

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Abstract: Problem statement: Palm oil mill effluent discharged by the oil palm industries is considered as the mixed of high polluted effluent which is abundant (about 20 million tonnes year^{-1}) and its effect contributes to the serious environmental problems through the pollution of water bodies. **Approach:** The aim of this study was to identify the potential of low cost substrate such as Palm Oil Mill Effluent (POME) for the production of cellulase enzyme by liquid state bioconversion. The filamentous fungus *Trichoderma harzianum* was used for liquid state bioconversion of POME for cellulase production. Statistical optimization was carried out to evaluate the physico-chemical parameters (factors) for maximum cellulase production by 2-level fractional factorial design with six central points. The polynomial regression model was developed using the experimental data including the effects of linear, quadratic and interaction of the factors. The factors involved were substrate (POME) and co-substrate (wheat flour) concentrations, temperature, pH, inoculum and agitation. **Results:** Statistical analysis showed that the optimum conditions were: Temperature of 30°C, substrate concentration of 2%, wheat flour concentration of 3%, pH of 4, inoculum of 3% and agitation of 200 rpm. Under these conditions, the model predicted the enzyme production to be about 14 FPU mL^{-1}. Analysis Of Variance (ANOVA) of the design showed a high coefficient of determination (R^2) value of 0.999, thus ensuring a high satisfactory adjustment of the quadratic model with the experimental data. **Conclusion/Recommendations:** This study indicates a better solution for waste management through the utilization of POME for cellulase production that could be used in the industrial applications such as bioethanol production.

Key words: Palm oil mill effluent, cellulase enzyme, bioconversion, statistical optimization

INTRODUCTION

Currently, Malaysia produces 15 million tonnes of crude palm oil per year from which about 12 million tonnes of palm oil is exported, indicates about 52% of the total world production (Wu *et al*., 2009). The process to extract oil from the Fresh Fruit Bunch (FFB) requires large amount of water, mainly for sterilizing the fruits and oil clarification, resulting in the discharge of organic, non-toxic wastewater known as Palm Oil Mill Effluent (POME). The quantity of POME produced is about 60% for every tonne of FBB processed. Thus, about 18-19.5 tonnes effluent (POME) h^{-1} is generated from the milling process of an average of 30 tonnes FBB h^{-1} (Rashid *et al*., 2009).

Several techniques have been developed in order to treat the highly biodegradable POME. Ponding, anaerobic and aeration systems are the most adopted treatment processes practiced by more than 85% of the palm oil mills in the country (Ma and Ong, 1985). The drawbacks of these systems are the requirement of a large land area and the system suffers from control and maintenance problems, biogas generation caused in air pollution (Ahmad *et al*., 2003). The production of this effluent always contributes an environmental problem such as the generation of methane during its anaerobic treatment and the production of high COD (Yacob *et al*., 2005). It is estimated that POME contains 95-96% water, 0.6-0.7% oil, 4-5% total solids, 2-4% suspended solids, pH 4.7, BOD-25000 mg L^{-1}, COD-50000 mg L^{-1} and total nitrogen-750 mg L^{-1} (Rashid *et al*., 2009). The carbohydrate and other nutrients content in POME effluent enable it to serve as substrate for the production of cellulolytic enzymes by liquid state bioconversion besides its treatment.

Cellulolytic enzyme production has attracted a world-wide attention due to the possibility of using this

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enzyme complex for conversion of abundantly available renewable lignocellulosic biomass for production of carbohydrates for numerous industrial applications including bioethanol (Gadjil *et al*., 1995; Xia and Cen, 1999). Economical production of cellulases is key for feasible bioethanol production from lignocellulosic biomass using cellulase-based processes. To date the production of cellulase has been widely studied in submerged culture processes (liquid state) but the relatively high cost of enzyme production has hindered the industrial applications through various expensive media are used to produce such enzymes (Reczey *et al*., 1996). The aim of this study was to determine the fermentation conditions by applying the statistical technique utilizing Palm Oil Mill Effluent (POME) as new substrate for the production of cellulase enzyme.

MATERIALS AND METHODS

Fermentation media: The major substrate/media used in this study was Palm Oil Mill Effluent (POME). The POME of 5-6% w/w of TSS (pH 4.8) was collected from oil palm industry named Seri Ulu Langat Palm Oil Mill Sdn. Bhd., Dengkil, Selangor, Malaysia. The POME concentration of 0.5-2% w/w of TSS was prepared by removing the excess water. The final pH of POME was recorded. The POME was supplemented with co-substrate of 1-3% w/w of wheat flour as available nutrients for microbe throughout the study (Alam *et al*., 2003).

Microbial strain and its inoculum preparation: Culture of *Trichoderma harzianum* was obtained from lab stock, Bioenvironmental Engineering Lab, IIUM. The fungus was maintained on Potato Dextrose Agar (PDA) plates and subcultured once in a month. The fungus was cultured onto the agar, incubated at 32°C until the entire plate was covered by fungus. After seven days, 100 mL sterilized water was poured onto the surface of four agar plates containing the spore culture. The spores on the surface were gently scraped with sterilized glass rod. The spore (3.2×10^5) suspension of *Trichoderma harzianum* was then filtered into a 250 mL Erlenmeyer flask and collected for further experiments.

Experimental procedure for cellulase production: The co-substrate of 1-3% (w/v) of wheat flour was added into POME with 0.5-2.0% (w/v) of TSS. The pH of the mixture was adjusted to 4-6 by adding HCl to increase acidity or NaOH to increase alkalinity. The mixture was sterilized at 121°C for 30 min. The inoculum of 1-3% with (3.2×10^5) spores was added into the flask and shaken in the shaker at agitation speed of 100-250 rpm. The pH and temperature was set at 4-6 and 30-35°C respectively during fermentation process. Sampling was done after four days of fermentation for the analysis of cellulase enzymes.

Table 1: Levels of experimental factors for optimization

Factor	Low (-1)	Center (0)	High (+1)
Temperature°C	30.0	32.50	35
Substrate conc (%)	0.5	1.25	2
Co-substrate conc (%)	1.0	2.00	3
Agitation, rpm	100.0	175.00	250
pH	4.0	5.00	6
Inoculums size (%)	1.0	2.00	3

Experimental design and optimization: Fractional factorial design with six center points was performed in order to determine the optimal fermentation conditions for the production of cellulase enzyme by *Trichoderma harzianum*. The experiments were conducted according to the designated two-level factorial design. In this design, six factors (parameters) with three levels (low, high and medium) were considered for determining the total number of experiments for optimization of cellulase production by liquid state bioconversion. The experiment was design using Minitab^{TM} statistical software. The factors with their levels are shown in Table 1.

Statistical and analytical analysis: Statistical software, Minitab^{TM} was used to analyze regression model of experimental data. The value of F-test, p-test, t-test and R^2 were identified to evaluate the model as well as to determine the optimum conditions. Cellulase activity assay was carried out by the method suggested by Ghose (1987).

RESULTS

The statistical optimization approach using fractional factorial design was used to study the linear, quadratic and interactive effects of various parameters on higher cellulase production by *Trichoderma harzianum.* The observed (experimental) and predicted results for cellulase enzyme production were obtained with different conditions is shown in Table 2. The highest cellulase activity (13.44 FPU mL^{-1}) was observe at run number 23, where the factors were found to be temperature 30°C, substrate concentration 2% and co-substrate concentration 3%, pH 4, inoculum size 3% and agitation 250 rpm. The experimental result was then analyzed by regression analysis, which gave the following regression Eq. 1 of the levels of cellulase produced (FPU mL^{-1}) as a function of temperature (x_1),

Table 2: Result using two level fractional factorial design and six center points showing observed and predicted response (cellulase)

Run	Temperature (°C)	Subs conc. (%)	Co-s conc. (%)	pH	Inoculum size (%)	Agitation (rpm)	Cellulase, FPU mL^{-1} Experimental	Predicted
1	30.0	0.5	1	4	1	100	3.277	2.673
2	35.0	0.5	1	4	1	250	2.643	1.941
3	30.0	2.0	1	4	1	250	2.643	2.194
4	35.0	2.0	1	4	1	100	7.400	6.848
5	30.0	0.5	3	4	1	250	2.696	2.118
6	35.0	0.5	3	4	1	100	2.273	1.540
7	30.0	2.0	3	4	1	100	0.000	-0.490
8	35.0	2.0	3	4	1	250	3.859	3.243
9	30.0	0.5	1	6	1	250	0.000	-0.578
10	35.0	0.5	1	6	1	100	3.753	2.984
11	30.0	2.0	1	6	1	100	0.000	-0.486
12	35.0	2.0	1	6	1	250	1.797	1.225
13	30.0	0.5	3	6	1	100	10.360	9.678
14	35.0	0.5	3	6	1	250	5.550	4.749
15	30.0	2.0	3	6	1	250	12.421	11.900
16	35.0	2.0	3	6	1	100	0.000	-0.676
17	30.0	0.5	1	4	3	250	1.850	1.349
18	35.0	0.5	1	4	3	100	5.286	4.661
19	30.0	2.0	1	4	3	100	0.000	-0.362
20	35.0	2.0	1	4	3	250	4.863	4.431
21	30.0	0.5	3	4	3	100	6.343	5.861
22	35.0	0.5	3	4	3	250	2.326	1.750
23	30.0	2.0	3	4	3	250	13.443	13.048
24	35.0	2.0	3	4	3	100	0.439	-0.034
25	30.0	0.5	1	6	3	100	7.559	7.063
26	35.0	0.5	1	6	3	250	2.114	1.568
27	30.0	2.0	1	6	3	250	3.330	2.994
28	35.0	2.0	1	6	3	100	1.216	0.774
29	30.0	0.5	3	6	3	250	2.167	1.673
30	35.0	0.5	3	6	3	100	5.920	5.263
31	30.0	2.0	3	6	3	100	0.000	-0.374
32	35.0	2.0	3	6	3	250	6.449	5.993
33	32.5	1.5	2	5	2	175	1.586	0.836
34	32.5	1.5	2	5	2	175	1.691	0.836
35	32.5	1.5	2	5	2	175	1.427	0.836
36	32.5	1.5	2	5	2	175	1.269	0.836
37	32.5	1.5	2	5	2	175	1.004	0.836
38	32.5	1.5	2	5	2	175	1.163	0.836

Table 3: ANOVA for polynomial model derived from experimental data

Source	DF	SS	MS	F	P
Regression	31	419.100	13.519	237.40	0.000
Residual error	6	0.324	0.057		
Total	37	419.442			

R^2: 0.999; R^2-adj: 0.995

SS: Sum of Squares; DF: Degree of Freedom; MS: Mean Square

substrate concentration (x_2), co-substrate concentration (x_3), inoculums size (x_4), pH (x_5) and agitation (x_6):

$$\begin{aligned}\text{Cellulase (FPU mL}^{-1}) = {} & 155-17.0x_1-89.4x_2+50.4x_3+59.2x_4+ \\ & 77.2x_5+0.0841x_6+0.382x_1^2+2.66x_1x_2- \\ & 1.69x_1x_3-1.79x_1x_4-2.30x_1x_5- \\ & 0.00450x_1x_6+9.82x_2x_3+2.31x_2x_4+ \\ & 0.830x_2x_5+0.326x_2x_6-7.91x_3x_4- \\ & 6.61x_3x_5+0.0204x_3x_6-12.9x_4x_5- \\ & 0.0979x_4x_6+0.00218x_5x_6\end{aligned} \quad (1)$$

The ANOVA from the analysis are shown in Table 3. The results showed that the coefficient of determination (R^2) was 0.999 and R^2-adj was 0.995 which ensured satisfactory adjustment of the quadratic model to the experimental data. The value of adjusted determination coefficient is also very high indicating a high significance of the model. The F-value is a measure of variation of the data about the mean. The high F-value and very low probability ($p>F = 0.0000$) indicates that the present model is in good prediction of the experimental result. The t-and p-values for the single, quadratic and interactive effects on the response are shown in Table 4 to evaluate the significance levels ($p<0.01$ or $p<0.5$).

DISCUSSION

The t-value and probability value (p-value) serves as a tool for checking the significant of each of the coefficient.

Table 4: t-value and p-value for each coefficient of the parameters and their interactions

Predictor	Coefficient	t	P
Constant	15535.0000	7.07	0.000
Temp, x_1	-17.0000	-14.43	0.000
Subs conc, x_2	-89.4100	-19.41	0.000
Co-s conc, x_3	50.3520	14.81	0.000
pH, x_4	59.1670	25.92	0.000
Inoculum, x_5	77.2390	24.52	0.000
Agitation, x_5	0.0841	2.00	0.092
x_1^2	0.3825	22.32	0.000
x_1x_2	2.6573	18.81	0.000
x_1x_3	-1.6900	-16.20	0.000
x_1x_4	-1.7880	-25.53	0.000
x_1x_5	-2.3010	-23.82	0.000
x_1x_6	-0.0045	-3.49	0.013
x_2x_3	9.8247	13.40	0.000
x_2x_4	2.3059	3.14	0.020
x_2x_5	0.8301	1.13	0.301
x_2x_6	0.3262	33.36	0.000
x_3x_4	-7.9104	-14.38	0.000
x_3x_5	-6.6121	-12.02	0.000
x_3x_6	0.0204	2.79	0.032
x_4x_5	-12.8990	-23.45	0.000
x_4x_6	0.0979	-13.35	0.000
x_5x_6	0.0022	3.88	0.008

The pattern of interactions between the variables is indicated by these coefficients. The larger the magnitude of t-test value and smaller the p-value indicates the high significance of the corresponding coefficient. The variable with low probability levels contribute to the model, whereas the other can be neglected and eliminated from the model. The p-value suggest that the coefficient for linear effect of temperature, x_1, substrate concentration, x_2, co-substrate concentration, x_3, inoculums size, x_4 and pH, x_5, are most significant with value of $p<0.000$ (Table 4). The p-value of the coefficient of interactive effect of substrate concentration and agitation (x_2x_6) had value of 0.0000, which is highly significant. Thus, from this result, it was clear that through the linear effect of agitation was not highly significant for cellulase production from *Trichoderma harzianum*, its addition to the design could not be totally overruled because of its interactive effect with wheat flour.

CONCLUSION

The results show that the optimum conditions for maximum cellulase production (about 14 FPU mL^{-1}) using palm oil mills effluent by liquid state bioconversion was found to be the temperature, 30°C; substrate concentration, 2% (w/v); co-substrate concentration, 3% (w/v); pH, 4; inoculum size, 3% (w/v) and agitation, 250 rpm. The optimization study of fermentation conditions using two-level fractional factorial design would enhance the production of enzymes at different levels using POME as substrate. The lab-scale study on cellulase production from POME as major substrate might give the basic information of further development for large scale production.

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