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Liquid State Bioconversion of Palm Oil Mill Effluent for Cellulase Production: Statistical Optimization of Process Conditions

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Abstract

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. Statistical analysis showed that the optimum conditions were: temperature of 30^0C, 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. Analysis of variance (ANOVA) of the design showed a high coefficient of determination (R^2) value of 0.99, thus ensuring a high satisfactory adjustment of the quadratic model with the experimental data.

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

1.0 Introduction

Currently, Malaysia produces 11.9 million tonnes of crude palm oil per year from which about 8.9 million tonnes of palm oil is exported, accounting for 52% of the total world production [1]. The process to extract oil from the fresh fruit bunch (FBB) requires large amount of water, mainly for sterilizing the fruits and for 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, an average of 30 tonnes FBB/hr mill in this country is generated about 18-19.5 tonnes effluent (POME)/hr [2].

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 [3]. 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 etc [4]. The production of this effluent always contributes an environmental problem such as the generation of methane during its

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anaerobic treatment and the production of high COD [5]. It is estimated that POME contain 95-96% water, 0.6-0.7% oil, 4-5% total solids, 2-4% suspended solids, pH 4.7, BOD-25000mg/L, COD-50000mg/L and total nitrogen-750mg/L [2]. 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 enzyme complex for conversion of abundantly available renewable lignocellulosic biomass for production of carbohydrates for numerous industrial applications including bioethanol [6-8]. 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 application of cellulose bioconversion through various expensive media are used to produce such enzymes [9]. The aim of this study was to determine the process conditions utilizing palm oil mill effluent (POME) as substrate which is abundant in Malaysia (about 10 million tonnes per year) for the production of cellulase enzyme by liquid state bioconversion.

2.0 Materials and Methods

2.1 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 have been prepared by removing excess water. This was done by leaving the sample for a few days to let the heavy particle to settle down at the bottom of the pail. Then a certain amount of excess water was removed according to the material balance to maintain the actual TSS in the sample (POME). 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 [10].

2.2 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 month. PDA was prepared by dissolving 9.75 g/L of PDA powder (OXOID Ltd. England) in 240mL of distilled water, sterilize for 20 minutes in 121°C autoclave before poured it into the plates to prepare for the solid PDA. Then the fungus was cultured onto the agar, incubated at 32°C until the entire plate was covered by fungus. After seven days, 100mL sterilized water was poured onto the surface of four agar plates containing the spore culture. The spore on the surface was gently scraped with sterilized glass rod. The spore ($2.8 – 3.2 \times 10^5$) suspension of *Trichoderma harzianum* was then filtered into a 250mL Erlenmeyer flask and collected for further experiments.

2.3 Experimental Procedure for Cellulase Production

1-3% (w/v) of wheat flour was added into flask containing 0.5%-2.0% (w/v) of POME. The pH of the mixture was adjusted to 4-6 by adding HCl to increase acidity or NaOH to increase alkalinity. The mixture was steam sterilized at 120°C for 30 minutes. 1% - 3% of inoculums with ($2.8 – 3.2 \times 10^5$) spores was added into the flask and shaken in the shaker with agitation speed of 100 -250 rpm. The pH and temperature was set at 4.0 – 6.0 and 30 - 35°C respectively during fermentation process. Sampling was done after four days of fermentation, enzyme activities was analyzed and measured.

2.4 Experimental Design and Optimization

Fractional factorial design with six center points was performed in order to determine the optimal process 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, total number of experiments and also level for each factor was determined. The experiments studied six factors for optimization of cellulase production by liquid state bioconversion. The experiment was design using Minitab™ statistical software. The levels and factors are shown in Table 1.

Table 1 Levels of experimental factors for optimization

Factor	Low(-1)	Center(0)	High(+1)
Temperature	30°C	32.5°C	35°C
Substrate concentration	0.5%	1.25%	2.0%
Co-substrate concentration	1%	2%	3%
Agitation	100 rpm	175 rpm	250 rpm
Initial pH	4	5	6
Inoculums size (v/v)	1%	2%	3%

2.5 Statistical and Analytical Analysis

Statistical software, Minitab™ 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. Each factor has two levels, high and low including central points, and each experiment is run in three replications. The fractional factorial design with central points including actual values is shown in Table 2. The enzyme activities, TSS, COD as well as the pH were analyzed. The TSS as biosolids and chemical oxygen demand (COD) were determined according to the standard methods [11]. Cellulase activity assay was carried out by the method suggested by Mandel et al. [12].

3.0 Results and Discussion

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) was observe at run number 23, where the factors temperature, substrate and co-substrate concentration, inoculums size, pH and agitation were

used at their level of low 30°C, high 2%, high 3%, high 3%, pH 4 and high 250 rpm respectively. The experimental result was then analyzed by regression analysis, which gave the following regression Equation (1) of the levels of cellulase produced (FPU/ml) as a function of temperature (x_1), substrate concentration (x_2), co-substrate concentration (x_3), inoculums size (x_4), pH (x_5) and agitation (x_6).

Table 4.1. Result using two level fractional factorial design and six center points showing observed and predicted response (cellulase)

Run	Temp, ^{0}C	Subs conc (%)	Co-s conc (%)	pH	Inoculum Size (%)	Agitation rpm	Cellulase, FPU/ml	Cellulase, Predicted
1	30	0.5	1	4	1	100	3.277	2.673
2	35	0.5	1	4	1	250	2.643	1.941
3	30	2	1	4	1	250	2.643	2.194
4	35	2	1	4	1	100	7.400	6.848
5	30	0.5	3	4	1	250	2.696	2.118
6	35	0.5	3	4	1	100	2.273	1.540
7	30	2	3	4	1	100	0.000	-0.490
8	35	2	3	4	1	250	3.859	3.243
9	30	0.5	1	6	1	250	0.000	-0.578
10	35	0.5	1	6	1	100	3.753	2.984
11	30	2	1	6	1	100	0.000	-0.486
12	35	2	1	6	1	250	1.797	1.225
13	30	0.5	3	6	1	100	10.360	9.678
14	35	0.5	3	6	1	250	5.550	4.749
15	30	2	3	6	1	250	12.421	11.900
16	35	2	3	6	1	100	0.000	-0.676
17	30	0.5	1	4	3	250	1.850	1.349
18	35	0.5	1	4	3	100	5.286	4.661
19	30	2	1	4	3	100	0.000	-0.362
20	35	2	1	4	3	250	4.863	4.431
21	30	0.5	3	4	3	100	6.343	5.861
22	35	0.5	3	4	3	250	2.326	1.750
23	30	2	3	4	3	250	13.443	13.048
24	35	2	3	4	3	100	0.439	-0.034
25	30	0.5	1	6	3	100	7.559	7.063
26	35	0.5	1	6	3	250	2.114	1.568
27	30	2	1	6	3	250	3.330	2.994
28	35	2	1	6	3	100	1.216	0.774
29	30	0.5	3	6	3	250	2.167	1.673
30	35	0.5	3	6	3	100	5.920	5.263
31	30	2	3	6	3	100	0.000	-0.374
32	35	2	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

$$FPU/ml = 155 - 17.0*x_1 - 89.4*x2 + 50.4*x3 + 59.2*x4 + 77.2*x5 + 0.0841*x6 + 0.382*x1^2 + 2.66*x1*x2 - 1.69*x1*x3 - 1.79*x1*x4 - 2.30*x1*x5 - 0.00450*x1*x6 + 9.82*x2*x3 + 2.31*x2*x4 + 0.830*x2*x5 + 0.326*x2*x6 - 7.91*x3*x4 - 6.61*x3*x5 + 0.0204*x3*x6 - 12.9*x4*x5 - 0.0979*x4*x6 + 0.00218*x5*x6 - 0.297*x1*x2*x3 - 0.0942*x1*x2*x4 - 0.0273*x1*x2*x5 - 0.00892*x1*x2*x6 + 0.262*x1*x3*x4 + 0.199*x1*x3*x5 - 0.000208*x1*x3*x6 + 0.383*x1*x4*x5 + 0.00296*x1*x4*x6 \quad (1)$$

The ANOVA from the analysis are shown in Table 3. The results showed that the coefficient of determination (R-sq) was 99.9% 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 [13,14]. 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 [15]. The F-value, and p- and t-value are shown in Table 3 and 4 respectively.

Table 3 ANOVA for polynomial model derived from experimental data

Source	**DF**	**SS**	**MS**	**F**	**P**
Regression	31	419.1	13.519	237.40	0.000
Residual error	6	0.324	0.057		
Total	37	419.442			

R^2 = 0.999, R-adj=99.5%, SS, sum of squares; DF, degree of freedom; MS. Mean square

The t-value and probability value (p-value) serves as a tool for checking the significant of each of the coefficient. 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 [16]. The variable with low probability levels contribute to the model, whereas the other can be neglected and eliminated from the model. The p-value for the linear, quadratic and polynomial terms is shown in Table 4. 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 0.000. The value of probability for coefficient of linear effect of agitation, x6, was quite high (0.092). This means that approximately 90.8% of the model affected by this variable. In contrast the p-value of the coefficient of interactive effect of substrate concentration and agitation (x2x6) 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.

Table 4 t-value and p-value for the polynomial models

Predictor	Coef	SE Coef	t	p
Constant	15535	21.96	7.07	0.000
Temp, x1	-17.004	1.179	-14.43	0.000
Subs conc, x2	-89.410	4.606	-19.41	0.000
Co-s conc, x3	50.352	3.401	14.81	0.000
pH, x4	59.167	2.283	25.92	0.000
Inoculums, x5	77.239	3.151	24.52	0.000
rpm, x5	0.08407	0.04200	2.00	0.092
x1*x1	0.38238	0.01713	22.32	0.000
x1*x2	2.6573	0.1413	18.81	0.000
x1*x3	-1.6896	0.1043	-16.20	0.000
x1*x4	-1.78785	0.07003	-25.53	0.000
x1*x5	-2.30089	0.09661	-23.82	0.000
x1*x6	-0.004497	0.001288	-3.49	0.013
x2*x3	9.8247	0.7334	13.40	0.000
x2*x4	2.3059	0.7334	3.14	0.020
x2*x5	0.8301	0.7334	1.13	0.301
x2*x6	0.326193	0.009778	33.36	0.000
x3*x4	-7.9104	0.5500	-14.38	0.000
x3*x5	-6.6121	0.5500	-12.02	0.000
x3*x6	0.020441	0.007334	2.79	0.032
x4*x5	-12.8988	0.550	-23.45	0.000
x4*x6	0.097878	0.007334	-13.35	0.000
x5*x6	0.0021810	0.0005625	3.88	0.008
x1*x2*x3	-0.29690	0.02250	-13.20	0.000
x1*x2*x4	-0.09424	0.02250	-4.19	0.006
x1*x2*x5	-0.02733	0.02250	-1.21	0.270
x1*x2*x6	-0.0089226	0.0003	-29.74	0.000
x1*x3*x4	0.29232	0.01687	15.55	0.000
x1*x3*x5	0.19886	0.01687	11.78	0.000
x1*x3*x6	-0.0002081	0.000225	-0.93	0.391
x1*x4*x5	0.38257	0.01687	22.67	0.000
x1*x4*x6	0.0029567	0.0002250	13.14	0.000

4.0 Conclusion

It can be concluded that the optimum conditions for maximum cellulase production (14 FPU/ml) using palm oil mills effluent by liquid state bioconversion was the substrate concentration, 2.0% (w/v); temperature, 30°C; pH, 4; co-substrate concentration, 3.0% (w/v); inoculums size, 3.0% (w/v) and agitation, 250 rpm. The optimization study of process conditions using two-level fractional factorial design would enhance the production of enzymes at multi-stage level 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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