Optimization of the Nutrient Supplients for Cellulase Production with the Basal Medium Palm Oil Mill Effluent

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Abstract**—**A statistical optimization was studied to design a media composition to produce optimum cellulolytic enzyme where palm oil mill effluent (POME) as a basal medium and filamentous fungus, *Trichoderma reesei* RUT-C30 were used in the liquid state bioconversion(LSB). 2% (w/v) total suspended solid, TSS, of the POME supplemented with 1% (w/v) cellulose, 0.5%(w/v) peptone and 0.02% (v/v) Tween 80 was estimated to produce the optimum CMCase activity of 18.53 U/ml through the statistical analysis followed by the faced centered central composite design(FCCCD). The probability values of cellulose (<0.0011) and peptone (0.0021) indicated the significant effect on the production of cellulase with the determination coefficient (R^2) of 0.995.

Keywords**—**Face centered central composite design (FCCCD), Liquid state bioconversion (LSB), Palm oil mill effluent, *Trichoderma reesei* RUT C-30.

I. INTRODUCTION

MALAYSIA produces and exports the highest quantity of palm oil products in the world. Malaysia produced 15.8 million tons of palm oil in the year 2006 alone. It is estimated that 0.5–0.7 tons of POME is discharged from the mill for every tons of oil palm fresh fruit bunches [1] in which about 40 million tons of POME from 372 mills was discharged in the year of 2004 [2]. Since the effluent discharge rate is very high, this industry has also been considered as one of the most potential sources of organic pollutants to the environment. Large quantities of solid and liquid wastes are generated in the form of empty fruit bunches, pericarp fibres, palm shells, palm kernel cake and palm oil mill effluent (POME) during the processing of oil palm fruits to produce oil. Microorganisms from relevant environments are capable of degradation and utilization of organic waste during their metabolism as well as secretion of secondary metabolites i.e. bio-products [3]. Therefore, bioconversion of POME is considered to be a useful measure to produce the natural product like - cellulase enzyme as POME consists of favourable nutrient composition – water (95-96%), oil (0.6-0.7%), total suspended solids (4-5%) and considerable amount of minerals. The different oil palm wastes have been reported to produce fertilizer, fuel, water reclamation, citric acid, cellulase enzyme etc [4-9] through bioconversion.

Cellulase enzymes provide a key opportunity for achieving tremendous benefits of biomass utilization [10] through the bioconversion of the most abundant cellulosic wastes into the simplest carbohydrate monomer, glucose. Cellulase enzymes, exocellobiohydrolase and endoglucanase, have wide applications in the textile, paper and pulp and feed industries. The most well-known application is the use of cellulases in biostoning. Heikinheimo et al. [11] showed that T. reesei-purified cellulase, EGII, was the most effective at removing color from denim, producing a good stonewashing effect with the lowest hydrolysis level. In the treatments of cellulose-containing textile materials during their manufacturing and finishing, cellulase enzymes are used [12]. Endoglucanases are also important for the degradation of b-glucan in feed which lowers the viscosity of the intestinal contents and hence the quality of the feed is improved [13].

The production of cellulases using various substrates and nutrients by microorganisms has been reported [14, 15]. Most of the fungi produce several enzymes with similar cellulolytic activity. *Trichoderma* releases at least four types of endoglucanases, two cellobiohydrolases [16] and three endoxylanases [17]. Rashid et al. [9] already studied the production of cellulase enzymes using the POME as a basal medium. However the yield didn't show much commercial potentiality due to the low productivity. The main challenge of an economical bioconversion process is to search and identify the potential microorganisms and suitable media composition for higher productivity. Carbon source and its pretreatment is the major cost contributing factor for the commercial enzyme production [18]. Cellulase enzyme production with expensive media constituents- celluclast, glucose, yeast extract, peptone, urea, KH2PO4, (NH4)2SO4, MgSO4, FeSO4, MnSO4, CoCl2, CaCl2 etc have been reported by many researchers [19, 20]. Therefore, by conducting systematic screening

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studies using POME as a basal media, optimum composition of the co-substrates can be selected in an effort to reduce the production cost of cellulase enzyme production, since POME is considered as a waste with minimal cost. In the current study, screening and selection of co-substrates, TSS of POME and mineral composition was conducted on the basis of cellulases production through the bioconversion of POME using the statistical experimental design.

II. Materials and Methods

A. Sample Collection

POME with 3.5-4 % (w/v) TSS was collected from Sime-Darby, Banting, Kuala Lumpur. The samples were taken into 20 liter washed & cleaned container. pH and temperature reading was taken on the site and TSS was determined of the collected fresh samples. Then the samples were stored at 4°C for further use.

B. Fungal Inoculum Preparation

Trichoderma reesei RUT C-30 (ATCC 56765) was used to optimize the bioconversion media constituents [9]. The strain was cultured on the PDA plate as inocula source and incubated at 30°C for 8-10 days until the good sporulation was observed. After maturation each plate was washed with 25 ml of sterile water and then filtered the spore suspension with Whatman no.1 filter paper. All these works have to be done under aseptic condition. The inoculum then kept at 4°C. This preparation of inoculum was made sure to have the spore concentration of $1.5 – 3 \times 10^8$ spores/ml.

C. Bioconversion Media Preparation

5% (v/v) of the fungal inoculum of the spore suspension was used into the bioconversion medium where POME was used as the basal medium along with the co-substrates and minerals. pH of the medium was adjusted initially at 4.8 by using 1M HCl and 1M NaOH before sterilization at 121^0C for 30 minutes. The bioconversion process for the optimum cellulase production was carried out in the 100ml Erlenmeyer flask with the working volume of 50ml for 5days at 30^0C and 150rpm.

D. Extraction and Analytical Method

Samples of fermented broth after 5 days of fermentation were filtered using Whatman no. 1 filter paper and the filtrate was assayed for total cellulase and endoglucanase activity [21]. The total cellulase activity was determined by filter paper assay (FPase) using Whatman No. 1 filter paper strip with the dimension 1.0 9 6.0 cm equivalent to 50 mg as a substrate and Carboxymethyl cellulose (CMC) was used as a substrate to measure the endoglucanase activity by carboxymethyl cellulase assay (CMCase). FPA cellulase (U/ml) is the derived unit for FPase and CMC cellulase (U/ml) is derived for CMC assay. One FPA is the concentration of cellulase that can release 2.0 mg of glucose from 50 mg of cellulose over a 60 min period and one CMC is the concentration of enzyme that can release 0.5 mg of glucose from 0.5 ml of the substrate CMC in 30 min. TSS was determined according to the standard method of APHA [22].

E. Experimental Design

Plackett-Burman Design

In the first step of the optimization process placket-burman experimental design was used to identify the significance of the ingredients of the media for the optimum production of cellulase enzyme. Plackett-Burman statistical experimental design is very useful and widely employed in the screening of major constituents of the media [23]. This design gave an output of 12 experimental runs (combinations) with 11 independent variables (Table 1). All the experiments were performed in triplicate and the average of cellulase activity was used as the response (dependant variable). The main effect of each variable was calculated as the difference between the average of measurements made at the high value (+) and at the low value (-). This model does not describe the interaction among the factors and it is used to evaluate and select the important factors that influence the response. The parameters selected for the experiment were TSS of POME, Cassava powder, wheat flower, sugar, cellulose, peptone, $(NH_4)_2SO_4$, KH_2PO_4, tween 80, $MnSO_4.H_2O$ and $MgSO_4.7H_2O$.

TABLE I PLACKETT-BURMANN DESIGN FOR THE SCREENING OF MEDIA

Run	A	B	C	D	E	F	G	H	J	K	L	FPA	CMC
	(%, w/v)											(U/ml)	
1	0.5	0	0	0	0	0	0	0	0	0	0	0.166	0.506
2	2	0	2	0	0	0	0.5	0.25	0.2	0	0.02	0.004	0.060
3	2	0	2	2	0	0.5	0	0	0	0.05	0.02	0.020	0
4	2	0	0	0	2	0.5	0.5	0	0.2	0.05	0	0.037	0.062
5	2	2	0	2	2	0	0.5	0	0	0	0.02	0.018	0.012
6	0.5	0	2	2	2	0	0.5	0.25	0	0.05	0	0.915	5.718
7	0.5	2	0	0	0	0.5	0.5	0.25	0	0.05	0.02	0.390	0.896
8	2	2	0	2	0	0	0	0.25	0.2	0.05	0	0.069	0.081
9	0.5	2	2	0	2	0	0	0	0.2	0.05	0.02	1.884	6.954
10	0.5	2	2	2	0	0.5	0.5	0	0.2	0	0	0.641	1.257
11	2	2	2	0	2	0.5	0	0.25	0	0	0	0.017	0.084
12	0.5	0	0	2	2	0.5	0	0.25	0.2	0	0.02	2.907	6.5

A, TSS of POME; B, Cassava powder; C, wheat flower; D, sugar; E, cellulose; F, peptone; G, $(NH_4)_2SO_4$; H,KH_2PO_4; J, tween 80; K, $MnSO_4.H_2O$; L, $MgSO_4.7H_2O$; The (+) indicates the high and (-) indicates the low level.

One factor at a time (OFA T) Design

The influences of TSS of POME, sugar, cellulose, peptone, KH_2PO_4, tween 80, and $MgSO_4.7H_2O$.on the production of cellulase enzyme were investigated with the one-factor- at-a-time (OFAT) method to observe the possible optimum levels of the parameters. The range of the each parameter was investigated in different level by changing the value alone [23].

Face Centered Central Composite Design

The face centered central composite design (FCCCD) under the response surface methodology (RSM) was employed in order to illustrate the nature of the response surface in the experimental region and elucidate the optimal conditions of the most significant independent variables. Two major variables namely peptone and cellulose concentraation were included in this model. The factors were examined at three different levels (low, basal, high) coded (-1, 0, +1) as shown in Table 2. According to the FCCCD for the three variables, 13 experimental runs (5 runs at centre point) were executed and their observations were fitted to the following second order polynomial model:

$$Y=\beta_0+\beta_1A+\beta_2B+\beta_{11}A^2+\beta_{22}B^2+\beta_{12}AB$$

where, Y is the dependent variable (cellulase production as response); A and B are the independent variable (peptone and cellulose concentration); β_0 is the regression coefficient at center point; β_1 and β_2 are the linear coefficients; β_{11} and β_{22} are the quadratic coefficients and β_{12} is the second order interaction coefficient. The developed regression model was evaluated by analyzing the values of regression coefficients, analysis of variance (ANOVA), p- and F-values. The quality of fit of the polynomial model equation was expressed by the coefficient of determination, R2. The statistical software package Design-Expert 6.0.8 (Stat Ease Inc., Minneapolis, USA) was used to identify the experimental design as well as to generate a regression model to predict the optimum combinations considering the effects of linear, quadratic and interaction on cellulase enzyme production. A final experiment was conducted to validate the FCCCD developed model.

TABLE II CODED AND ACTUAL VALUES OF THE FACTORS FOR FCCCD ALONG WITH THE EXPERIMENTAL AND PREDICTED VALUES OF CELLULASE ENZYME ACTIVITY

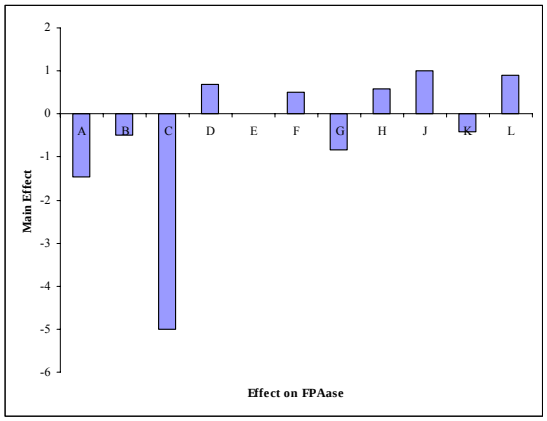
Run	A:Peptone(%, w/v)	B:Cellulose(%, w/v)	CMCase (U/ml)	
			Experimental	Predicted
1	0.2(-)	0.2(-)	6	6.3
2	0.8(+)	0.2(-)	10.5	10.42
3	0.2(-)	0.8(+)	13.5	13.53
4	0.8(+)	0.8(+)	8.9	8.55
5	0.2(-)	0.5(0)	13.7	13.37
6	0.8(+)	0.5(0)	12.5	12.94
7	0.5(0)	0.2(-)	13.26	13.04
8	0.5(0)	0.8(+)	15.4	15.72
9	0.5(0)	0.5(0)	18	17.84
10	0.5(0)	0.5(0)	17.9	17.84
11	0.5(0)	0.5(0)	17.52	17.84
12	0.5(0)	0.5(0)	17.75	17.84
13	0.5(0)	0.5(0)	18.13	17.84

III. RESULTS AND DISCUSSION

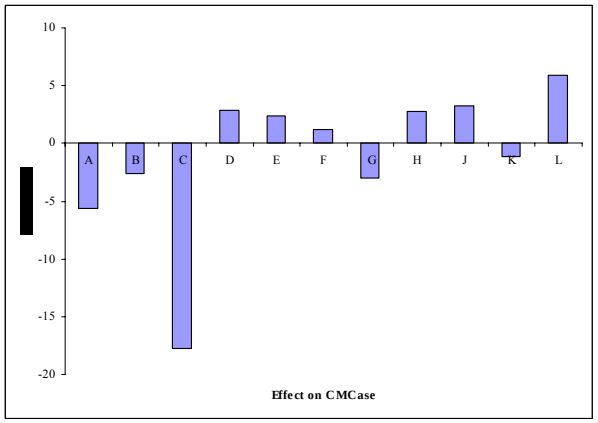
A. Evaluation of media constituents

Plackett-Burman design has been employed to evaluate the significant effect of the eleven different co-substrates and mineral elements on the production of cellulase using POME as a basal medium. The main effect of each constituent on the cellulase production was calculated as the difference between the average measurement calculated at the higher (+) and lower (-) levels of the constituent. The results, Fig. 1, showed that sugar, cellulose, peptone, KH_2PO_4, tween 80 and $MgSO_4.7H_2O$ has the positive effect on both of the parameters (FPAase and CMCase) of the cellulase production .On the other hand TSS of POME, cassava powder, wheat flower, $(NH_4)_2SO_4$ and $MnSO_4.H_2O$ has the negative effect on the both type of activity of cellulase. There is no report of positive influence of cassava and wheat powder on the production of the cellulase enzyme alone. Minerals' effect on enzyme production is dependant on it's concentration in the bioconversion medium [24].

The consistency of the influences on each type of the activity would be helpful to decide which parameters should be evaluated in the next phase of study. Cassava powder, wheat flower, $(NH_4)_2SO_4$ and $MnSO_4.H_2O$ was excluded from the constituents list of medium due to their negative effect on the production. TSS of POME was considered to be investigated to further studies even though it showed reverse effect on production, since it is impossible to exclude.



a. Main effects on FPAase



b. Main effects on CMCase

Fig. 1. Main effects of the medium constituents on cellulase production from Plackett-Burman experimental results (A: TSS (POME), B: cassava, C: wheat, D: sugar, E: cellulose, F: peptone, G: $(NH_4)_2SO_4$, H: KH_2PO_4, J: tween 80, K: $MnSO_4.H_2O$, L: $MgSO_4.7H_2O$).

B. Determination of optimum range of the media constituents

Seven constituent parameters out of eleven were screened by placket-burman design to determine the effective range of parameters for cellulase production by

one-factor-at-a-time (OFAT) method. Sugar, cellulose, peptone, KH_2PO_4, tween 80, $MgSO_4.7H_2O$ and TSS of POME was considered to be studied by the OFAT method. Each of the parameters was varied at five levels of concentration.

During bioconversion with the varied concentration KH_2PO_4 and $MgSO_4.7H_2O$ did not show any considerable increase in production. KH_2PO_4 and $MgSO_4.7H_2O$ was dropped from the media composition because it was studied that inclusion of these minerals didn't show any contributory effect in the production of enzyme. TSS showed a reciprocating effect on the production. Lower TSS is not acceptable due to the requirement of huge volume of water. 2% TSS was fixed because the net change in the production is 10% following the change in TSS from 0.2%-2% (w/v).

Among the two carbon sources cellulose showed very strong positive effect between the ranges 0.5-1.5% (w/v). On the contrary, the other carbon source, sugar, gave very high negative impact on the production of the cellulase production, so it was excluded from the medium composition. Ahmed and vermette [25] and Domingues et al. [26] also found cellulose as a best carbon source to induce the production of cellulase enzyme.

Peptone gave a strong positive contribution to the production of cellulase in the range between 0.5-1.0% (w/v) as a extra nitrogen source to the basal medium. Tween 80 showed a negative effect on the response at the higher range, but it was observed good contribution to the production in the range 0.2-0.3% (v/v). Domingues et al (2000) described the influence of tween 80 on the production of cellulase and the morphology of the Trichoderma. Following the study of Reese and Maguire (1969) and the OFAT results it was decided to have the tween 80 concentration at 0.2% (v/v).

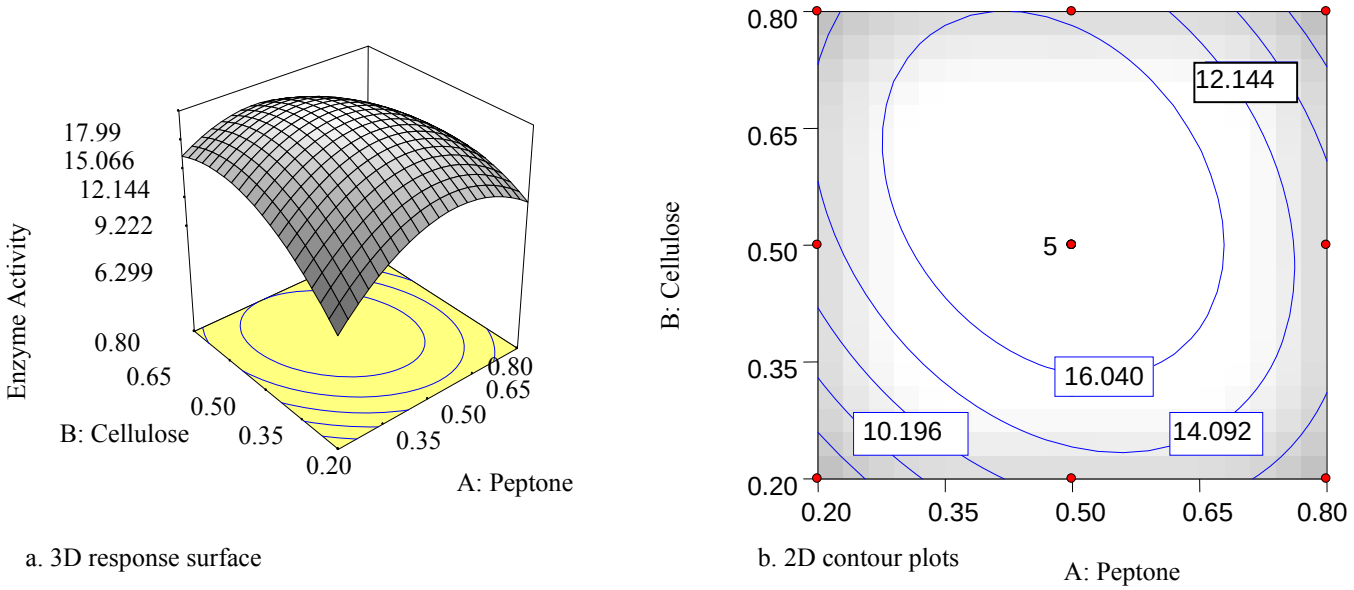
C. Statistical optimization of the media constituents

An experimental design, faced centered central composite design (FCCCD), was employed to optimize two independent variables, peptone and cellulose, as media constituents to maximize the production of cellulase by using the POME as a basal medium. A polynomial regression equation was developed under response surface methodology (RSM) to analyze factor interaction by identifying the significant factors contributing to the regression model and determine the optimal values f the most significant independent variables. The effects of two independent variables (cellulose and peptone) on cellulase production were predicted by the following polynomial regression equation-

Y (Cellulase activity, U/ml) = -12.97+63.97 * A+55.5 * B-52.1 * A^2-38.4 * B^2-25.3 * A * B

Where, the cellulase production in term of activity (Y) is a function of peptone (A) and cellulose (B).

The coefficient of determination (R^2) was 0.995 which ensure a satisfactory data and indicated that approximately 99.5% of the variability in the dependent variable, cellulase activity, could be explained by the model. The adjusted R^2 was 0.98, which is more suitable for comparing models with different numbers of independent variables.



a. 3D response surface

b. 2D contour plots

Fig. 2 Effect of peptone and cellulose concentration (%, w/v) on the production of cellulase enzyme (activity, U/ml)

The 3D response surface and 2D contour plots are the graphical representation of the regression equation used to determine the optimum values of the variables within the ranges considered [27]. Cellulase activity was higher with the

higher concentration of peptone and cellulose and decreased with the lower concentration of peptone and cellulose. The highest cellulase activity was predicted 17.84 U/ml on the center point with the concentration of 0.5% (w/v) of peptone and cellulase. The highest experimental values were also shown on the center points of the experimental design.

A set of experiment was performed to verify the optimization result to validate the developed model. Medium was composed with the parameters of the independent variables those were shown in the Table 3. The highest cellulase activity, 18.53 U/ml, was obtained in the optimum condition, which one is slightly higher than the predicted value, 17.84 U/ml. It is evident from this study that the production of cellulase was optimized at around 18 U/ml with the medium constituent concentration of peptone and cellulase is 0.5% (w/v).

TABLE III VALIDATION OF DEVELOPED POLYNOMIAL EQUATION FOR OPTIMUM MEDIUM CONSTITUENT

Run	A:Peptone(%, w/v)	B:Cellulose(%, w/v)	Cellulase (U/ml)	
			Predicted	Experimental
1	0.3	0.6	16.47	16.3
2	0.4	0.6	17.71	17.42
3	0.5	0.5	17.84	18.53
4	0.6	0.4	16.67	17.55
5	0.7	0.3	14.19	13.37

IV. CONCLUSIONS

In this study, eleven constituents of the media were studied by three different experimental designs sequentially where POME was used as a basal medium. In the first stage 4 constituents of the medium were excluded through plackett-burman design and the rest 7 parameters were further studied following the OFAT method. In this method another 3 parameters were dropped from the medium composition, 2 were fixed and the rest 2 were optimized by the FCCCD under RSM finally. At last 4 parameters of the medium constituents were optimized to produce the optimum cellulase, 18.53 CMCase (U/ml). The final production, 18.53 CMCase (U/ml), is 2.7 times higher than the highest production, 6.95 CMCase (U/ml), obtained at the placket-burman experimental design.

ACKNOWLEDGMENTS

The authors thank to the Ministry of Higher Education (MOHE) for approval a research grant under Fundamental Research Grant Scheme (FRGS 0207-49). The co-operation of the Sime-darby, Banting, by supplying the experimental samples is acknowledged as well.

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