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Optimization of Media Composition for the Production of Bioprotein from Pineapple Skins by Liquid-State Bioconversion

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Abstract: Two-steps optimization strategy based on statistical experimental design was carried out to enhance bioprotein production by liquid state bioconversion utilizing pineapple skins as substrate. A two-level Plackett-Burman design was employed first where 11 variables were studied for their influence on bioprotein production. Pineapple Skins Soluble (PSS) sugar content, KH_2PO_4 and $NH_4H_2PO_4$ were the most significant variables for improving bioprotein production. A three-level Central Composite Design (CCD) was employed for maximizing the bioprotein production. A mathematical model was developed to show the effects of each medium component and their combinatorial interaction on bioprotein production. The optimal medium composition for maximum bioprotein production was PSS content 1.0%, KH_2PO_4 0.1% and $NH_4H_2PO_4$ 0.25%. The final experiment was carried out to validate the model, which has shown to produce 514.2 g kg^{-1} bioprotein on the 5th day of fermentation with a slight increase of 0.02% from the experimental run in CCD.

Key words: Bioprotein, liquid state bioconversion, pineapple skins, Plackett-Burman, central composite design

INTRODUCTION

Demand for nutritious food has been increased year by year as world population growth increased, leading to a massive starvation and malnourished people. Agro-based industries such as pineapple canning industry are one of the major liquid and solid waste producers in Malaysia, although it plays important roles in the countries socio-economic conditions after palm oil and rubber industry (Roslina, 2008). Healthy life and cleaner environment is the end result of solving these problems in such a way by utilizing the waste into valuable by-products. These wastes can be regarded as new sources for bioprotein production, which have a high nutritional value, do not compete with food for human consumption, economically feasible and locally available (Anupama and Ravindra, 2000).

Bioprotein is the protein extracted from cultivated microbial biomass which can be used as protein supplementation for human and animal (Jamal *et al*., 2007). For humans, it is also considered as food additive to improve flavor, fat binding and more recently as a replacement for animal protein in the diet. Bioproteins can be produced using a number of different substrates such as low carbon cost, high energy sources and agricultural wastes (Uysal *et al*., 2002). Agricultural wastes or food processing wastes such as pineapple waste has been found to be potential to be used as raw material for conversion into useful value added-products. Pineapple waste, which constituted of 30% after canning operation, contains high content of carbohydrate that can be utilized as carbon source for the production of organic acid (Roslina, 2008). Algae, fungi and bacteria are the chief sources of microbial protein that can be utilized as a protein (Jamal *et al*., 2007). Many fungal species are used as a protein-rich food. They provide the B-complex group of vitamins and thy also show a low level of nucleic acid content. Biomass obtained from *P. chrysosporium* has been found to contain most of the essential amino acids (Balagopalan and Padmaja, 2000).

Fermentation has been identified as one of less expensive means of increasing the protein quality of low cost carbohydrate such as cassava and wheat flour (Ghaly *et al*., 2004). Bioprotein can be produced successfully from fermentation of carbon sources by suitable microorganism using either liquid state bioconversion or solid state bioconversion approach. The proper utilization of a continuous flow biological unit for bioprotein production depends on understanding of its kinetics as well as the effects of the operating parameters

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(Srinivasan, 1979). Optimization of process conditions could increase the amount of protein having high nutritional value and will have no competition with food for human consumption. Thus, production of microbial proteins or bioproteins by fermentation of agricultural waste products is one of the most promising approaches for increasing the availability of proteins in the world (Jamal *et al*., 2006).

Based on the current trends of treating the waste such as bioconversion to value added products, this study was inspired to develop an optimized media utilizing pineapple skins for the production of value added product which is bioprotein by microbial treatment.

MATERIALS AND METHODS

Media and inoculum preparation: The pineapple skins were washed and cut into smaller pieces (1-2 cm) before grinding with distilled water (1:1 (w/v)) and their original concentration were determined. *Phanerochaete chrysosporium* PC-13 (basiodomytes) was obtained from the lab stock. The inoculum was prepared by washing the growing culture in four PDA plates with 100 mL sterile distilled water and their spore suspension were rubbed prior to filter using filter paper Whatman No. 1 into 250 mL Erlenmeyer flask (Anupama and Ravindra, 2000).

Bioprotein production by liquid state bioconversion: The production of bioprotein by liquid state bioconversion was carried out in 100 mL Erlenmeyer flasks containing 50 mL media as per experimental design (Table 1, 4). The medium used was composed of 2.5% pineapple skins as the carbon source with the initial pH 4.5. Initial PSS content was measured using total sugar method by Dubois *et al.* (1956), nitrogen sources and trace elements were varied according to the statistical experimental design. The inoculated flasks were incubated in incubator shaker at 150 rpm at 32°C for 5 days. All runs were replicated three times and the bioprotein was estimated. The biomass analysis was done according to APHA (APHA/AWWA/WPCH, 1989), where the biomass was dried in oven at 50-60°C for 48 h before cooling in desiccators. Total protein and total sugar concentration were measured according to Lowry *et al.* (1951) method using Folin-Phenol Reagent and total sugar method (Dubois *et al*., 1956), respectively.

Optimization of media composition using Plackett-Burman design and Central Composite Design (CCD): Two experimental designs were carried out for optimization of media composition, which were the Plackett-Burman design and the CCD. Experimental designs were generated by using the statistical software package Design Expert 6.0.8 Stat-Ease, Minneapolis, MN and the statistical analysis of experimental data was also performed using this software.

Table 1: Plackett-Burman design for 11 parameters with coded values along with the observed results

Runs	A	B	C	D	E	F	G	H	I	J	K	Bioprotein (g kg^{-1})
1	1	-1	1	-1	-1	-1	1	1	1	1	-1	337.44
2	1	1	-1	1	1	-1	1	-1	-1	1	-1	337.67
3	1	1	1	-1	1	1	-1	1	-1	-1	-1	353.62
4	1	-1	1	1	-1	1	-1	-1	-1	1	1	339.07
5	1	1	-1	1	-1	-1	-1	1	1	-1	1	322.55
6	1	-1	-1	-1	1	1	1	-1	1	-1	1	320.25
7	-1	1	1	1	-1	1	1	-1	1	-1	-1	330.54
8	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	264.89
9	-1	1	1	-1	1	-1	-1	-1	1	1	1	340.83
10	-1	-1	-1	1	1	1	-1	1	1	1	-1	327.09
11	-1	-1	1	1	1	-1	1	1	-1	-1	1	310.03
12	-1	1	-1	-1	-1	1	1	1	-1	1	1	315.01

Variables are listed in alphabetical order and their levels are given in (%w/v), A: PSS content (0.5-1.0), B: KH_2PO_4 (0.05-0.25), C: $NH_4H_2PO_4$ (0.05-0.25), D: $MgSO_4.7H_2O$ (0.05-0.1), E: $FeSO_4$ (0.001-0.005), F: $ZnSO_4$ (0.001-0.005), G: $CaCl_2$ (0-0.001), H: $MnSO_4$ (0-0.001), I: $CuSO_4$ (0-0.001), J: NaCl (0-0.001), K: dummy variable

Plackett-Burman design: The Plackett-Burman experimental design is a valuable tool for the rapid evaluation of the effects of various medium components. It can pick up the main factors with the least number of experiments from a list of candidate factors (Plackett and Burman, 1946; Kalil *et al*., 2000). The Plackett-Burman experimental design based on the first-order model in Eq. 1:

$$Y = \beta_0 + \sum \beta_i x_i \quad (1)$$

where, Y is the response (bioprotein production g kg^{-1}), β_0 is the model intercept and β_i is the linear coefficient and x_i is the level of the independent variable (Plackett and Burman, 1946).

Because this design is a preliminary optimization technique, which tests only on two levels of each medium component, it cannot provide the optimal quantity of each component required in the medium.

The screening of most significant fermentation parameters affecting bioprotein production was investigated by the Plackett and Burman (1946) design. The media component comprises of carbon and nitrogen source as well as trace elements. A total of 11 parameters comprising ten variables such as Pineapple Skins-Extracted Sugar (PSS) content, KH_2PO_4, $NH_4H_2PO_4$, $MgSO_4.7H_2O$, $FeSO_4$, $ZnSO_4$, $CaCl_2$, $MnSO_4$, $CuSO_4$ and NaCl and one dummy variable were studied in 12 (11+1) trials (Table 1).

Each parameter was represented at two levels, low and high, denoted by (-1) and (1), respectively. The effect of each variable was determined by following Eq. 2:

$$E_{(xi)} = \frac{2(\Sigma M_{i+} - M_{i-})}{N} \quad (2)$$

where, $E(x_i)$ is the effect of the tested variable M_{i+} and M_{i-} are the bioprotein production from the trials where the variable (x_i) measured was present at high and low concentrations, respectively and N is the number of trials (Plackett and Burman, 1946).

Experimental error was estimated by calculating the variance among the dummy variables as follows in Eq. 3:

$$V_{eff} = \frac{\Sigma(E_d)^2}{n} \quad (3)$$

where, V_{eff} is the variance of the concentration effect, E_d is the concentration effect for the dummy variable and n is the number of dummy variables.

The Standard Error (SE) of the concentration effect was the square root of the variance of an effect and the significance level (p-value) of each concentration effect was determined using student's t-test (Eq. 4):

$$t_{(xi)} = \frac{E_{(xi)}}{SE} \quad (4)$$

where, $E(x_i)$ is the effect of variable x_i.

Central Composite Design (CCD): A second round of factor level selection was conducted using the Central Composite Design (CCD) as to investigate the optimum value and to study the interactions of the most effective parameters (PSS content, KH_2PO_4 and $NH_4H_2PO_4$), screened by preliminary experimental design, the Plackett-Burman design. Each parameter in the design was studied at three different levels, with all variables taken at a central coded value of zero (Table 2).

Total number of 19 experiments, with an axial point (α = 1.0) and five replicates at the center points was employed (Table 4). For three parameters system, the model equation is as follows in Eq. 5:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 \quad (5)$$

Table 2: Central composite design levels

		Levels		
Parameters	Units	-1	0	+1
PSS content (X_1)	% w/v	0.5	1.0	1.5
KH_2PO_4 (X_2)	% w/v	0.1	0.25	0.4
$NH_4H_2PO_4$ (X_3)	% w/v	0.1	0.25	0.4

RESULTS

Optimization of media composition using experimental design (Plackett-Burman design for selection of significant media components): Screening and selection of significant media components was done by Plackett-Burman design (Table 1) and had shown the importance of optimizing media composition in attaining higher production of bioprotein. Among 10 parameters, three factors (PSS content, KH_2PO_4 and $NH_4H_2PO_4$) were found to have the confidence level above 95% (Table 3) and hence were considered to affect the bioprotein production significantly on the basis of carbon and nitrogen source used for the growth of microorganism.

The optimal conditions of these parameters were further measured by using the Central Composite Design (CCD) with nineteen experiments (Table 4). Other parameters such as $FeSO_4$, $ZnSO_4$, $CuSO_4$ and NaCl were trace elements also found to be significant (confidence level >95%) and these parameters were not varied in CCD design but included in optimization by maintaining their high level value. These trace elements were

Table 3: Statistical calculations for Plackett-Burman design

Sources	Effect	t-value	p-value	Confidence level (%)
PSS content (A)	20.37	70.24	0.0182	98.18
KH_2PO_4 (B)	16.91	58.31	0.0219	97.81
$NH_4H_2PO_4$ (C)	20.68	71.30	0.0180	98.20
$MgSO_4$ (D)	5.82	20.06	0.0636	93.64
$FeSO_4$ (E)	13.33	45.97	0.0278	97.22
$ZnSO_4$ (F)	12.03	41.47	0.0308	96.92
$CaCl_2$ (G)	0.48	1.67	0.5594	44.06
$MnSO_4$ (H)	5.42	18.68	0.0683	93.17
$CuSO_4$ (I)	9.74	33.58	0.0381	96.19
NaCl (J)	15.87	54.74	0.0234	97.66

Table 4: Experimental design and result of CCD of response surface methodology for the optimization of bioprotein production

Runs	PSS (% w/v) X_1	KH_2PO_4 (% w/v) X_2	$NH_4H_2PO_4$ (% w/v) X_3	Bioprotein production (g kg^{-1})
1	1.0	0.40	0.25	485.06
2	1.5	0.10	0.10	403.01
3	1.0	0.10	0.25	514.09
4	1.0	0.25	0.25	509.13
5	1.0	0.25	0.10	473.89
6	1.0	0.25	0.25	509.13
7	1.0	0.25	0.25	509.13
8	0.5	0.10	0.10	434.17
9	1.0	0.25	0.25	509.13
10	1.0	0.25	0.25	509.13
11	1.5	0.40	0.10	364.25
12	1.5	0.40	0.40	378.85
13	0.5	0.40	0.40	401.76
14	0.5	0.10	0.40	409.07
15	1.5	0.25	0.25	438.69
16	0.5	0.40	0.10	402.69
17	1.0	0.25	0.40	468.64
18	0.5	0.25	0.25	465.73
19	1.5	0.10	0.40	393.43

Table 5: ANOVA for quadratic model

Sources	Sum of square	df	Mean square	F-value	p>F
Model	48349	9	5372.16	1547.7	0.0001
Residual	31.24	9	3.47		
Lack of fit	31.24	5	6.25		
Pure error	0.00	4	0.00		
Total	48381	18			

$R^2 = 0.9994$, CV = 0.41, Adj. $R^2 = 0.9987$

essential in supporting and enhancing the growth of *P. chrysosporium* as well as to promote the production of bioprotein.

Central Composite Design (CCD): The coefficients of the response surface model were evaluated by regression analysis and tested for their significance. The coefficient of determination (R^2) of the model is 0.9994 and the model F-value is 1547.68 (Table 5), which indicates the model to be suitable to represent adequately the real relationship among the parameters used. The final predictive equation obtained is given by Eq. 6:

$$\begin{aligned}\text{Bioprotein } (\text{g kg}^{-1}) = {} & 510.18 - (13.52 \times \text{PSS}) - (12.12 \times KH_2PO_4) \\ & - (2.63 \times NH_4H_2PO_4) - (59.29 \times \text{PSS}^2) \\ & - (11.93 \times KH_2PO_4^2) - (40.23 \times NH_4H_2PO_4^2) \\ & - (1.82 \times \text{PSS} \times KH_2PO_4) + (3.88 \times \text{PSS} \times NH_4H_2PO_4) \\ & + (6.04 \times KH_2PO_4 \times NH_4H_2PO_4)\end{aligned} \quad (6)$$

The theoretically calculated response time value using Eq. 6 was found to be in good agreement with experimentally determined values. The signal-to-noise ratio (108.3) was desirable. All linear term of parameter (X_1, X_2, X_3), the parameter interactions (X_1X_2, X_1X_3, X_2X_3) and square of the parameters (X_1X_1, X_2X_2, X_3X_3) used in this study are significant, which are shown in Table 6.

Experimental validation of medium optimum conditions obtained with the central composite design: An experimental run was carried out to validate the model and to determine the maximum production of bioprotein under the optimal value of 1.0% (w/v) PSS, 0.10% (w/v) KH_2PO_4 and 0.25% (w/v) $NH_4H_2PO_4$. The maximum production of bioprotein obtained was 514.2 g kg^{-1} on the fifth day of fermentation (Fig. 1) and it was found to be 0.02% more than the experimental run obtained at similar media condition.

The soluble sugar content from the substrate was found to be utilized gradually and decreased from 9.7 to 0.76 g L^{-1} throughout the fermentation days, as the bioprotein production increased in response with the growth of *P. chrysosporium* PC-13. The growth of the microorganism started to decline, since the carbon source was depleted constantly starting on the 6th day of fermentation.

Table 6: Model coefficient, as estimated by multiple linear regressions

Model terms	Parameter estimate	SE	Computed t-value	p-value
Intercept	510.18	0.68	748.00	<0.0001*
PSS (X_1)	-13.52	0.59	-22.94	<0.0001*
KH_2PO_4 (X_2)	-12.12	0.59	-20.56	<0.0001*
$NH_4H_2PO_4$ (X_3)	-2.63	0.59	-4.46	0.0016*
X_1X_1	-59.29	1.13	-52.60	<0.0001*
X_2X_2	-11.93	1.13	-10.58	<0.0001*
X_3X_3	-40.23	1.13	-35.70	<0.0001*
X_1X_2	-1.82	0.66	-2.76	0.0221*
X_1X_3	3.88	0.66	5.89	0.0002*
X_2X_3	6.04	0.66	9.17	<0.0001*

*Significant at 5% level ($p<0.05$)

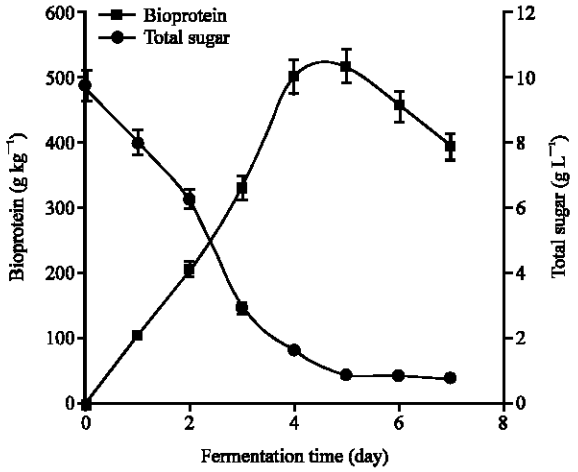

Fig. 1: Bioprotein production by *P. chrysosporium* with optimized media

DISCUSSION

Potential and suitable media for bioprotein production utilizing pineapple skins as substrate by liquid state bioconversion has been identified and optimized by two set of optimization design. Selection of medium in the first optimization design was highlighted by the significant effect of PSS as carbon source, $NH_4H_2PO_4$ and KH_2PO_4 as inorganic nitrogen source, the main components in the growth of biomass and building block of proteins.

Although, some other parameters like $FeSO_4$, $ZnSO_4$, $CuSO_4$ and NaCl were also found to be significant, these medium were not varied in next optimization but included as supplementation to the media at their high level value. These parameters can be regarded as mineral salts or micronutrients to stimulate the growth of *P. chrysosporium* PC-13, as their requirement are less than carbon and nitrogen sources.

The significant parameters were varied in second optimization, in which their optimal value and interaction effects were studied. In response to the bioprotein production, all three parameters had shown significant

effect on their linear, quadratic and interactions terms. The importance of using carbon and nitrogen source were reported to be essential in providing a suitable growth media for *P. chrysosporium* which has been found through research work on the molasses wastewater (MWW) (Ahmadi *et al*., 2006), while there are also many reports on use of C and/or N sources for support of *P. chrysosporium* growth (Kirk *et al*., 1978; Jansheker and Fiechter, 1983). Soluble sugar from pineapple skins as cheaper carbon source was used in this research as its potential, low cost and high content of carbohydrate, make it more suitable compared to other commercially sugar in the market. Moderate amount of sugar (PSS) and $NH_4H_2PO_4$ as carbon and nitrogen source, respectively were proven to increase the bioprotein production 1.5 times from maximum production of 353.62 g kg^{-1} using Plackett-Burman design to 514.09 g kg^{-1} using central composite design.

The effect of nitrogen source in bioprotein production utilizing pineapple skins as substrate was shown to be important beside the carbon source as the primary nutrient. The amount of yeast biomass produced in the fermentation medium containing only pineapple juice from pineapple skins as carbon and nitrogen sources were lower than those with nitrogen supplementation (Rosma and Cheong, 2007). This is due to the lack of nitrogen sources in this juice (Rosma and Cheong, 2007) and upon using pineapple wastes as the substrate, additional nitrogen source is required to support both microbial and biomass production. This is also reported by Nancib *et al*. (2001) that production of lactic acid by using date juice as fermentation medium could be increased by supplementing date juice with nitrogen sources. The effect of inorganic nitrogen source was reported by Chung and Muhammad (2007) to give the highest yield in cellulose production. Therefore, the use of and KH_2PO_4 and $NH_4H_2PO_4$ as inorganic nitrogen sources to the media for *P. chrysosporium* PC-13 growth were showed to play a significant role to maximize bioprotein production, as it produced 514.24 g kg^{-1} along with PSS as carbon source in the final validation experiment.

CONCLUSION

In the present study, optimum media composition was determined for the production of bioprotein using *P. chrysosporium* PC-13 by using the Plackett-Burman design and CCD. The Plackett-Burman statistical design technique was found to be useful for identifying the most influential components of the system (PSS content, KH_2PO_4 and $NH_4H_2PO_4$). The second optimization design, CCD was used to check interactions and concentration of significant media components. About a 0.2% increase in bioprotein production was achieved on the fifth day of fermentation, after using statistical techniques for media optimization. A higher yield of bioprotein production by liquid state bioconversion using a lower concentration (%w/v) of media components (PSS -1.0, KH_2PO_4 - 0.10 and $NH_4H_2PO_4$ -0.25) is the major outcome of this study. Further studies are required to maintain the production conditions by controlling aeration, agitation and other physical parameters such as pH and temperature in the fermentor. The yield of bioprotein can also be increased by using pure oxygen to support the aeration in fermenter and to increase the growth of *P. chrysosporium*.

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