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MEDIA OPTIMIZATION FOR BIOPROTEINS PRODUCTION FROM CHEAPER CARBON SOURCE

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Abstract

There are high demands for animal and human food supply especially protein, which is an important dietary component. Agricultural wastes, cheap carbon sources- which are rich and have high energy, can be used for producing the value added bioprotein. A lab scale study was carried out to optimize the media composition for bioprotein production from a cheaper carbon source - wheat flour using potential strain, which was selected earlier by screening different microorganisms. The performance of the selected strain was enhanced by media optimization with varied substrate concentration, nitrogen sources and nutrient supplementation according to the central composite design from STATISTICA software. Statistical optimization was carried out to evaluate the polynomial regression model through effect of linear, quadratic and interaction of the factors. The maximum biomass produced was 21.89 g/L with optimum fermentation conditions of wheat flour (4 g/L), nitrogen concentration (0.5 g/L), nutrient concentration (0.1 g/L), and four days of fermentation.

Keywords: Media optimization, Bioprotein, Wheat flour

1. Introduction

The sustainable development of a country requires sustained production of food, fuel, and chemicals. Due to the shift from agricultural to electronics and other fast-paying industrial development, most of the countries around the world, including Malaysia, ceased to be self-sufficient in their food production - in particular protein, which is necessary to satisfy their optimum food demands [1, 2]. The biotechnology advancement and its application to agriculture have brought hope to them. The race

to catch up with the need for food by modernization of agriculture has gained much attention throughout the world. However, the necessity for exploring unconventional, non-agricultural means of food production, especially of proteins rich food, cannot be over-emphasized due to the burgeoning populations in several regions of the world, especially in its southern hemisphere [1]. In addition to this, the importance of protein in food nutrient cannot be neglected because its shortage causes various malnutrition problems. This situation has created a demand for the formulation of innovative and alternative proteinaceous food sources having high nutritional value [3]. These sources have to be non-competitive with food for human consumption, economically feasible and locally available.

Thus, the production of bioproteins (proteins derived from micro-organisms) by fermentation of agricultural waste products, with low cost carbon sources and high energy sources are the most promising breakthrough of biotechnological innovations [4]. This will certainly increase the availability of affordable quality proteins and reduce dependence on animal proteins. High production of bioproteins could be possible due to rapid growth rate of microorganisms such as algae, fungi and bacteria [5]. The bioprotein produced by them can be utilized as a protein supplement because they are quite rich in protein, carbohydrates, fatty acids, vitamins and minerals. Due to the increasing demand for bioproteins, the potential strain, substrate and optimum conditions must be investigated for a high-yield product.

In this study, we emphasized on media optimization to produce maximum quality bioprotein using liquid state bioconversion process by introducing cheaper carbon source- wheat flour as the substrate. This substrate is easily available in Malaysia, and have high nutritional value, high carbohydrate percentage, and available at low cost. A strain of *Mucor hiemalis* was used in this research to improve the bioprotein production, which was previously screened for its potentiality to produce maximum biomass, by comparing with four different microorganisms - *Aspergillus niger, Phanerochaete chrysosporium, Saccharomyces cerivisiea,* and *Thricoderma harzianum* [6]. The selected strain was further used (to maximize the production of bioproteins) for media optimization with varied concentration of the substrate, nitrogen sources and nutrient supplementation according to the central composite design from STATISTICA software. Five code levels ranging from -2 to 2 were considered for all factors in order to determine the response of different levels. Code level 0 is the central value obtained from preliminary experiments conducted in the lab while code levels -1, -2, 1 and 2 are lower and higher variations of the code level 0. Each experiment was performed in three replicates for accurate result. The values of pH, temperature, inoculum size, agitation and aeration were controlled during the experiment. Performance of the experiment was evaluated on the basis of biomass production and total protein.

2. Materials and Methods

2.1. Sample collection

Wheat flour (Alagappa's Atta Flour) was purchased from the local market. Sample was stored at room temperature for further use.

2.2. Fungal strain

Strain was obtained from the lab stock of IIUM. It was isolated on Malt Extract Agar (Merk, Germany) agar and purified on Potato Dextrose Agar (PDA) agar. Then, the strain was cultured and maintained on (PDA) slants and stored at 4ºC. Subculture was done once a month.

2.3. Inoculum preparation

Inoculum preparation (spore suspension) was done according to method suggested in [7]. The fungal strain was cultured on 3.9% potato dextrose agar (PDA) at 32ºC for 7 days and then transferred into Erlenmeyer flask (250 mL) using 100 mL of sterile distilled water. It was shaken in a rotary shaker at 150 rpm for 24 hours and then filtered. The filtrate was used as inoculum after measuring its concentration (spores mL^{-1}) by Haemocytometer. All flasks, funnel, filter paper, distilled water were sterilized prior to use.

2.4. Liquid state bioconversion (LSB)

Liquid state bioconversion was done in 500 mL Erlenmyer flasks using selected strain of *Mucor hiemalis*. The media compositions used in all experiments were based on the central composite design. Inoculum was added after sterilization at 121ºC for 20 minutes and other optimum process conditions were maintained in all experimental flasks. The experimental samples were analyzed after four days of fermentation. The effectiveness of the optimum media compositions were evaluated by bioprotein concentration determined according to the suggested method [8].

2.5. Biomass analysis

The biomass was filtered by vacuum filtration (pore size, 11 micrometer) and washed three times with 20 mL of distilled water. Before taking the weight of the biomass, it was transferred into an aluminum disk and dried in an oven at 103ºC-105ºC for one hour followed by cooling in desiccators to balance the temperature and weight [7].

2.6. Total protein determination

Protein determination was done according to Lowry method (Folin-Phenol Reagent) [9]. All reagents were prepared according to the suggested concentration and added to the sample solution as instructed in the method. Spectrophotometer reading was recorded at 660 nm after 20 minutes.

2.7. Experimental design and optimum process conditions

Design of the experiment and statistical analysis in this study was done using statistical software STATISTICA. The optimization was done using central

composite design with three factors; substrate, nitrogen and nutrient source and five levels for all parameters. The variables and their levels for central composite design are shown in Table 1. Regression analysis was done where multiple regression equations was developed and followed by analysis of the regression equation by statistical analysis; ANOVA (analysis of variance), regression coefficient (R^2), F-test (overall model significance), t-test and P-value (each coefficient significance).

Table 1. Factors and Levels Including Actual Values for Optimization of Media Compositions.

Factors	Symbol	Code Levels				
		-2	-1	0	+1	+2
Substrate concentration	X_1	0.5	1.5	2.5	3.5	4.0
Nitrogen sources concentration	X_2	0.0	0.05	0.1	0.15	0.2
Nutrient supplementation	X_3	0.0	0.05	0.1	0.15	0.2

The average maximum of biomass was taken as the dependent variable of response (Y). The data were plotted to develop a second order polynomial equation by multiple regression procedure. This resulted in an empirical model that relates the response measured due to in dependent variables of the experiment. For three factor system, the model polynomial equation is:

$$Y = f(X_1, X_2, X_3)$$

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

where Y is the biomass production (g/L), predicted response; β_0, intercept; $\beta_1, \beta_2, \beta_3$, linear coefficient; $\beta_{11}, \beta_{22}, \beta_{33}$, squared coefficients; $\beta_{12}, \beta_{13}, \beta_{23}$, interactions coefficients.

3. Results and Discussion

Design of experimental and statistical analysis

Based on the screening results, *M. hiemalis* was selected as the potential strain for the bioprotein production from wheat flour. Three factors were selected for optimization of media; wheat flour concentration (X_1), nitrogen concentration (X_2) and nutrient supplement concentration (X_3). The fermentation was done for four days according to CCD to find the optimum composition of the media to produce maximum bioprotein. A total of 16 treatments with three replications for each experiment were conducted (Table 2). The statistical analysis of the data was done by statistical software and the developed polynomial regression equation relating to the production of bioprotein with the three independent variables X_1, X_2

and X_3 are given below:

$$Y = -17.8 + 12.2X_1 + 23.8X_2 + 148X_3 - 1.31X_1^2 - 14.6X_2^2 - 486X_3^2 + 0.70X_1X_2 - 5.8X_1X_3 - 56X_2X_3 \quad (2)$$

The average (triplicate values) of biomass production was taken as the dependent variable or response Y. The combinations of independent variables along with experimental and predicted results of microbial biomass concentration are shown in Table 2.

Table 2. Values of Observed and Predicted Response and Protein Concentration.

Run	Wheat Flour (g/L) X_1	NH_4NO_3 (g/L) X_2	KH_2PO_4 (g/L) X_3	Protein. (g/kg)	Biomass Concentration (g/kg)	
					Observed	Predicted
1	1.5	0.2	0.05	5	7.308	7.1285
2	1.5	0.2	0.15	7	9.026	10.2185
3	1.5	0.4	0.05	12	8.99	9.7865
4	1.5	0.4	0.15	10	10.146	11.7565
5	3.5	0.2	0.05	13	19.18	18.1285
6	3.5	0.2	0.15	15	20.29	20.0585
7	3.5	0.4	0.05	15	21.7	21.0665
8	3.5	0.4	0.15	16	21.13	21.8765
9	0.5	0.3	0.1	14	3.249	1.8735
10	4	0.5	0.1	18	21.89	24.51
11	2.5	0.1	0.1	12	14.69	14.8515
12	2.5	0.5	0.1	9	20.53	19.3275
13	2.5	0.3	0	7	10.35	10.8635
14	2.5	0.3	0.2	6	16.322	14.7635
15	2.5	0.3	0.1	9	18.44	17.6735
16	2.5	0.3	0.1	18	18.368	17.6735

In order to obtain the best fit regression of the model, the regression equation and coefficient of determination (R^2) were evaluated. The model portrayed a good determination of coefficient (R^2 = 0.974) which explained that 97.4% of the variables (wheat flour, nitrogen and nutrient sources) attributed to the production. The high value of adjusted R^2 = 93.5 % also indicated a high significance of the model [10, 11].The corresponding of analysis of variance (ANOVA) is presented in Table 3, which demonstrated the significance of the model due to a very low probability value [($P_{model} > F$) =0.000].

Table 3. ANOVA for the Selected Quadratic Model.

Source	Degree of freedom	Sum of Squares	Mean Squares	*F*-value	*P>F*
Regression	9	530.857	59.984	24.88	0.000
Residual Error	6	14.223	2.371		
Total	15	545.081			

The student t-distribution and the corresponding p-values along with the second order polynomial coefficient were evaluated. The significance of each coefficient was determined by t-values and p-values which indicates the pattern of interactions between the variables. The variables with low probability levels contribute to the model, whereas the others can be neglected and eliminated from the model. The larger the magnitude of t-value and smaller the p-value indicate the high significance of the corresponding coefficient [12]. The t-values and p-values for the linear, quadratic and interactive terms are given in Table 4. The p-values were used to check the significance of each coefficient. The variables with low p-value (0.01-0.05) and the larger magnitude of test value indicated high significant correlation of coefficients.

From Table 4, it can be seen that the variables with the largest effect were the linear term of substrate (X_1), and nutrient (X_3), as well as the squared term of substrate (X_1X_1) and nutrient (X_3X_3). Furthermore, the interaction between substrate and ammonium (X_1X_2); substrate and nutrient (X_1X_3); and ammonium and nutrient (X_2X_3) did not show any major significant effect. Since the effect of substrate and nutrient are significant, they can act as limiting factor or nutrients and little variation in their values and concentration could contribute to the product formation in the process.

Table 4. Results of Regression Analysis of the Central Composite Design.

Predictor	Coefficient	Standard error coefficient	t-value	p-value
Constant	-17.755	8.122	-2.19	0.071
Substrate, X_1	12.234	3.105	4.06	0.007
Ammonium, X_2	23.75	28.95	0.82	0.443
Nutrient, X_3	147.73	52.83	2.80	0.031
X_1X_1	-1.3050	0.4757	-2.74	0.034
X_2X_2	-14.57	37.99	-0.38	0.715
X_3X_3	-485.7	152.0	-3.20	0.019
X_1X_2	0.698	5.444	0.13	0.902
X_1X_3	-5.83	10.89	-0.54	0.611
X_2X_3	-56.0	108.9	-0.51	0.625

From the statistical analysis, the highest biomass production predicted by the regression equation was in Run 10, which predicted a yield of 24.51 g/kg. It was found that within the given range, the optimal value for test variable were substrate (wheat flour) 4% w/v, nitrogen source (ammonium nitrate) 0.5% w/v and nutrient source (potassium dihydrogen phosphate) 0.1% w/v. The maximum dried bioprotein obtained experimentally by using the above optimal concentrations was 21.89 g/kg. This was only slightly less than the predicted value, thus showing that the proposed empirical model is accurate.

4. Conclusions

It can be concluded that the selected factors for media optimization have the profound effect on biomass production. The selected potential strain *M. hiemalis* predicted the highest biomass on fourth day of fermentation by using 4% w/v of substrate (wheat flour), 0.5% w/v of nitrogen source (ammonium nitrate) and 0.1% w/v nutrient source (potassium dihydrogen phosphate), which is higher compared to the experimental value. Further optimization of process conditions expected to improve the production rate, which is in progress. Furthermore, this study would be beneficial in producing large quantity of nutritional bioprotein from a cheaper carbon source, which can also contribute to fulfill the protein demand of the world's ever increasing population. Abundant supply of quality protein will certainly improve the quality of human and animal life on the earth, presently also in times to come. This can also be used as a supplement and additive in the animal feed. It can also be utilized as additives in certain chemical and pharmaceutical products.

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