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Response Surface Methodology for Mycoprotein Production by *Fusarium Venenatum* ATCC 20334

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Abstract

In this research, the effect of process variable on yield (%w/w protein per biomass) of mycoprotein production by *Fusarium venenatum* ATCC 20334 in surface culture evaluated. A face centered central composite design (FCCD) was employed to determine maximum protein production at suitable initial concentration of date juice (as a carbon and energy source), nitrogen concentration and seed size. Analysis of variance showed that the contribution of a quadratic model was significant for the response. The optimal condition for mycoprotein production contains 20 g/l of date juice, 4.48 g/l of nitrogen source and 12.97% (v/v) of seed size. In these conditions, 46.48 ± 0.2% (w/w) protein was obtained in the dried cell weight. Heat treatment of fungal biomass at 64 -65˚C for 20-30 min reduced the RNA content to an acceptable level for human food grade products. Finally, after reduction of ribonucleic acid contents of mycoprotein, the amino acids and fatty acids profiles of product were determined.

Keywords: *Fusarium venenatum*; Mycoprotein; Central Composite Design (CCD); Surface culture; Date juice

Introduction

Fusarium venenatum has been cultured as a mycoprotein source for human consumption in England for over a decade under the trade name of "Qourn". This product with a fibrous texture is a rich source of high quality protein including essential amino acids. It is also less energy dense than equivalent meat products and does not have animal fats and cholesterol [1]. Mycoprotein shows satiety and satiation properties which can be a solution for overweight by enabling people to achieve a healthier diet (low fat and high fiber) [2].

The most commonly used medium for biomass production by *F. venenatum* is Vogel medium with glucose as a carbon source [3]. Several economic substrate like agricultural wastes are been introduced as the carbon and energy sources for mycoprotein production, e.g. wheat starch (the substrate chosen by RHM company in England), potatoes (in Ireland), cassava, rice or cane juice (in tropical countries) [4].

Date fruit with high content of carbohydrates, minerals and vitamins, is produced in Middle East. Unfortunately a large amount of this product is wasted, while it is rich in carbohydrates and other required metabolites for microbial growth and production. Use of date as carbon and energy sources results cheap fermentation process due to less required pretreatment and low cost substrate. Date has been used for the production of baker's yeast biomass [5], lactic acid [6,7], alkaline protease [8], xantan [9], cultivation of mushrooms [10] and some other microbial metabolites.

Many studies have been conducted for mycoprotein production. Wiebe used *F. venenatum* A3/5 to produce mycoprotein in 150,000 liter pressure reactors in continues flow process on glucose and ammonium (as the carbon and nitrogen sources) [11]. Ahangi were used *F. oxysporum* for production of mycoprotein while this fungus shows allergic and toxic symptoms in consumer. The results showed that in optimum condition the dried fungal biomass contained 42%w/w protein and the productivity was still low (5g/l biomass contain of 42% protein) [12]. Also the results indicated that mechanical agitation damages mycelial biomass and reduces the yield of production [13]. Therefore, application of surface culture method for production of mycoprotein can be proposed to fill gap of research in this field.

The optimization of variables in mycoprotein production (as like as other fermentation processes), is of primary importance due to the impact on the feasibility and efficiency of the process. The first step is identification of the main process variables. The selection of the variables in this study was based on our prior experience by Plackett–Burman design (PBD) [14]. In addition, the rheological properties of fungal biomass from *F. venenatum* were determined in different conditions of temperature and shear [15]. Since the PBD is typically used as a preliminary optimization technique, more accurate quantitative analysis of the effect of variables for mycoprotein production is required [16]. Further optimization can be conducted by response surface methodology (RSM), a factorial base design introduced by G.E.P. Box in the 1950s [17]. RSM mainly includes central composite design (CCD), Box-Behnken design, one-factor design, D-optimal design, user defined design, and historical data design [18].

In this study, a face centered composite design (FCCD) was adapted to optimize the levels of medium variables (date juice and nitrogen source concentrations as well as seed size) on production of mycoprotein. This is the first time that date juice is used for protein production by *F. venenatum* ATCC 20334 in surface culture. The biomass was produced under determined condition. Finally, after reduction of ribonucleic acid contents of mycoprotein, the amino acids and fatty acids profiles of product were determined.

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Materials and Methods

Microrganism

F. venenatum ATCC 20334 was used throughout this investigation. Strain was maintained at 4˚C on agar-solidified Vogel slants. The components of modified Vogel medium is described elsewhere [14].

Inoculum and media preparation

For preparation of date juice, the wastage of date (prepared from Dombaz Company) was added to water and boiled in a 50 L tank for 30 min. The components of filtrate was determined [8] and used as carbon and energy source in inoculum development medium. Inocula were prepared in 250 conical flasks containing 50 mL Vogel medium, which were inoculated and incubated on a rotary shaker at 30˚C and 200 rpm for 72 h. Production medium also contained date juice as carbon source and other medium components were the same as in seed medium. Fermentation was conducted in 500 mL flasks each containing 100 mL of production medium. The culture medium was inoculated with fungal suspension, and incubated in surface culture at 30˚C.

After fermentation process, biomass was harvested by filtration of 100 mL cultivation medium through pre-dried Whatman No.1 filter papers. Then, clarified suspension was passed throw a 0.45 µm membrane, was washed twice with cold distilled water and dried using an oven at 60°C to a constant weight. The cell dry weight was quantified gravimetrically.

RNA reduction

The RNA content of biomass was reduced in order to meet required safety standards [11] by subjecting the biomass to heat shock at 64 -65 ˚C for 20-30 min.

Response surface methodology

The main and interaction of three variables which influence the mycoprotein production were analyzed and optimized by FCCD (α = 1) in three levels Table 1. A total of 20 experimental runs with different combination of variables (consisting 14 experimental runs and 6 additional runs at the center point) to check reproducibility were carried out. Protein production was taken as the response (Y). The general form of second order polynomial, which its coefficients were analyzed by Minitab 14, is as Equation1:

$$Y_i=\beta_0+\Sigma\,\beta_i X_i+\Sigma\,\beta_{ii}X_i^2+\Sigma\,\beta_{ij}X_iX_j \qquad \text{Eq (1)}$$

where Y_i is the predicted response, X_iX_j are input variables, which influence the response variable Y; β_0 is the offset term; β_i is the i^{th} coefficient; β_{ii} the j^{th} quadratic coefficient and β_{ij} is the ij^{th} interaction coefficient.

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The optimum levels of variables (within the experimental range) for maximum protein production were determined, and maximum protein production was confirmed by running trial number 10.

variables	Coded levels		
	-1	0	+1
Date juice (g/l)	10	15	20
$(NH_4)\,H_2PO_4$ (g/l)	3	4	5
Seed size (% v/v)	8	12	16

Table 1: Levels of independent variables in the experimental design for mycoprotein production.

Analytical methods

The crude protein content was measured using the Kjeldahl technique [19]. The crude protein content of the biomass was calculated by multiplying 6.25 to total nitrogen. The protein content was represented by %w/w protein per total dry weight. Determination of RNA content of biomass was performed by spectrophotometeric method [20]. Amino acid profile of *F. venenatum* was determined by Pico-Tag method. This method involves hydrolysis of biomass with HCL; derivatized with phenylisothiocyanate to produce phenylthiocarbamyl amino acids and analysis by reverse phase HPLC [21]. Fatty acid composition of biomass was determined by a Younglin ACME 6000M gas chromatograph (Korea) using a capillary column (Technokroma TR-CN 100) (60 m per 0.25 mm ID, film thickness 0.2 µm) operated with hydrogen as the carrier gas (0.2 ml/min). The operating conditions were as follows: injector temperature 250˚C; detector temperature 260˚C; split ratio 80:1, oven temperature program was 5 min at 150˚C, then increasing by 5˚C per min up to 175˚C then 3 min in this temperature followed by 3˚C per min up to 190˚C, and finally 15 min at 190˚C [22].

Results and Discussion

Regression model and statistical testing

The experimental central composite design was applied to analyze the main and interaction effects of date juice, nitrogen concentration and seed size. The optimum condition was obtained for mycoprotein production, as the design and results of trials are given in Table 2. Multiple regressions were used to analyze the data and thus polynomial equation was derived from regression analysis as follows:

$$Y=45.5063+0.9559\,X_1+0.4129\,X_2+0.3448\,X_3+0.0062\,X_1^2-0.5978\,X_2^2-0.6153\,X_3^2+0.0931\,X_1X_2-0.6706\,X_1X_3+0.8356\,X_2X_3 \qquad (2)$$

Regression analysis of the experimental data showed that date juice, nitrogen and seed size had positive linear effect on mycoprotein production ($P< 0.05$). Among the three variables date juice had highest impact on protein production as given by highest linear coefficient (0.9559), followed by nitrogen source (0.4127) and seed size (0.3448). Elimination of insignificant terms was done step by step. Date juice showed insignificant quadratic effect on protein production with maximum *P* value among other terms. Hence, in the first step this term was excluded from the regression and conclusions were repeated Table 3. All rest terms had significant effects on protein production by low *P* values (<0.05) as showed in Table 3. So the model Equation 2 was modified to reduced fitted model Equation 3:

$$Y=45.5071+0.9590\,X_1+0.4127\,X_2+0.3448\,X_3-0.5954\,X_2^2-0.6129\,X_3^2+0.0931\,X_1X_2-0.6706\,X_1X_3+0.8356\,X_2X_3 \qquad (3)$$

Analysis of variance (ANOVA) for the protein production obtained from this design is shown in Table 4. ANOVA gives the value of the model and can explain whether the model adequately fits the variation observed in protein production with the designed variable level. F test for regression was significant at a P<5%. The coefficient of determination (R^2) for production of mycoprotein was 99.5%. This value showed good agreement between experimental observations and predicted values. The coefficient of variation indicates the high degree of precision. The higher reliability of the experiment is usually indicated by low value of S. In the present case, a low value of S at 0.1146 denotes that the experiments performed are highly reliable. The *P* value for lack of fit was 0.236. This amount indicated that the experimental data obtained fitted well with the model and explained the effect of date juice and nitrogen

Run	Date Juice (g/l)(X_1)	(NH_4) H_2PO_4 (g/l)(X_2)	Seed size (% v/v) (X_3)	Protein (g/100)(Y)	
				experimental	predicted
1	-1	-1	-1	42.910	42.843
2	1	-1	-1	45.875	46.003
3	-1	1	-1	41.775	41.811
4	1	1	-1	45.290	45.251
5	-1	-1	1	43.210	43.203
6	1	-1	1	43.670	43.587
7	-1	1	1	45.595	45.513
8	1	1	1	46.250	46.270
9	-1	0	0	44.441	44.551
10	1	0	0	46.405	46.463
11	0	-1	0	44.378	44.499
12	0	1	0	45.260	45.324
13	0	0	-1	44.515	44.549
14	0	0	1	44.088	44.962
15	0	0	0	45.720	45.507
16	0	0	0	45.505	45.507
17	0	0	0	45.530	45.507
18	0	0	0	45.501	45.507
19	0	0	0	45.640	45.507
20	0	0	0	45.500	45.507

Table 2: Face centered composite design matrix for evaluation of the three variables and experimental and predicted mycoprotein production.

Variable	Coef.	SE Coef.	*t*-value	*P*-value[a]
constant	45.5071	0.03842	1184.423	0.000
X_1	0.9559	0.03622	26.389	0.000
X2	0.4127	0.03622	11.398	0.000
X3	0.3448	0.03622	9.519	0.000
X22	-0.5954	0.06404	-9.299	0.000
X32	-0.6129	0.06404	-9.572	0.000
X1X2	0.0931	0.04050	2.299	0.042
X_1X_3	-0.6706	0.04050	-16.559	0.000
X_2X_3	0.8356	0.04050	20.633	0.000

[a]Significant at P<0.05
S = 0.1146 R^2 = 99.5% R^2(adj) = 99.1%

Table 3: Coefficients and t-values of process variables for mycoprotein production using face centered composite design.

Source	Degree of freedom (DF)	Sum of squares (SS)	Mean of squares (MS)	$S_{tatistics}$	P-value[a]
Model	8	27.1240	3.39049	258.39	0.000
Linear	3	12.0295	4.00984	305.59	0.000
Square	2	5.8410	2.92049	222.57	0.000
Interaction	3	9.2534	3.08448	235.07	0.000
Residual error	11	0.1443	0.01312		
Lack of fit	6	0.1015	0.01692	1.98	0.236
Pure error	5	0.0428	0.00856		
Total	19	27.2683	-	-	

[a]Significant at P<0.05

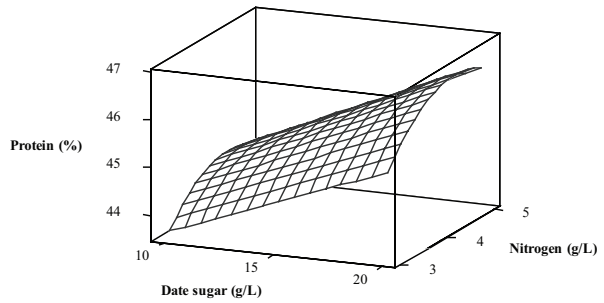
Table 4: Analysis of variance for mycoprotein production using face centered composite design.

source contents and seed size on mycoprotein production by *F. venenatum*. The insignificant value of lack of fit (more than 0.05) showed that the quadratic model was valid for the present study [23].

Interaction between factors influencing mycoprotein production

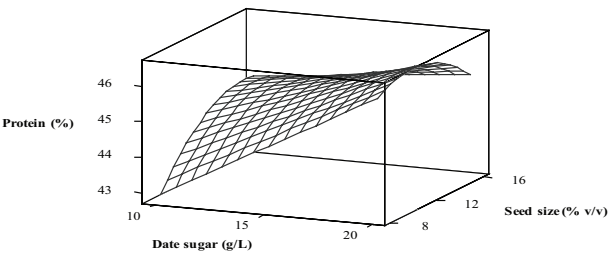
The interaction effects and optimal levels of the variables were determined by plotting the response surface curves. The effect of interaction of date juice and nitrogen on protein production is illustrated in Figure 1. The surface plot showed increasing trend for mycoprotein production with increased date juice and a moderate value of nitrogen. The maximum protein production was achieved by adding 20 and 4 g/L of sugar syrup and $NH_4H_2PO_4$ as carbon and nitrogen sources. In fact, increased concentration of produced protein is a result of increased carbon and nitrogen sources concentrations. Further increase of nitrogen concentration causes decreased production of mycoprotein. Carbon is the main component of cellular structure and energy storage. Nitrogen is a key factor in protein and nucleic acid production. In addition, $NH_4H_2PO_4$ plays a buffer role in medium. The initial sugar requirement for growth varies from species to species and from strain to strain [24].

Figure 2 represents the interaction between date juice and seed size. This Fig showed that intermediate level of seed size and high date juice favored mycoprotein production. Results indicate that an inoculum amount of 12% is the suitable inoculums size for protein production. Increase of inoculum size results increased protein production from date juice. Obviously, this observation is due to decreased lag phase and promoted cell growth in early stage of fermentation. Similar results has been reported by Jin et al. for protein production from starch waste by *Asperglilus oryzae*, who determined 7.5% (v/v) is optimum level of seed size in data range of 1 to 15.5% (v/v) [25]. Further increase of inoculum size with constant concentration of substrate may cause decreased production due to limitation of medium components and resulted compe-



Hold values: Seed size (%v/v) 12

Figure 1: Response surface of interaction effect between date juice and nitrogen source concentration on mycoprotein production.



Hold values: Nitrogen (g/L) 4

Figure 2: Response surface of interaction effect between date juice concentration and seed size on mycoprotein production.

tition between cells.

Figure 3 shows effect of nitrogen and seed size on mycoprotein production. High production of protein was observed at higher level of nitrogen and seed size. Therefore, increase of both variables enhances protein production.

Optimization and verification

The surface plots in Figure1-3 indicated that the produced protein in some regions could be more than 46.5%. Hence, the response optimization based on desirability function was carried with Minitab 14. The parameters given by response optimization for date juice, nitrogen and seed size consist of 20 g/L, 4.48 g/L and 12.97%, respectively. Under this condition protein production was 46.55%. Therefore, these conditions were used for confirmation of the predicted value of protein output. Maximum yield of protein production 46.48 ± 0.2%w/w was obtained under optimized experimental conditions.

Heat treatment at 64 -65˚C for 20-30 min reduces the RNA content from 7.88 to 0.9% which is a safe level for human consumption (maximum limit is 2 g/day). This result is in agreement with previous studies about *F. venenatum* [11] (Wiebe, 2002). Amino and fatty acids composition of fungal biomass are presented in Table 5 and 6. Table 5 shows that the amino acid profile of biomass include all the essential amino acids. This is in agreement with results reported by Rodger in 2001 [26]. Analysis of fatty acids profile indicated that the ratio of unsaturated to saturated fatty acid was 3.21 to 1 Table 6. Rodger also has reported ratio of 3.5 to 1 for unsaturated to saturated fatty acids. High amounts of unsaturated fatty acids may cause several benefits for human health.

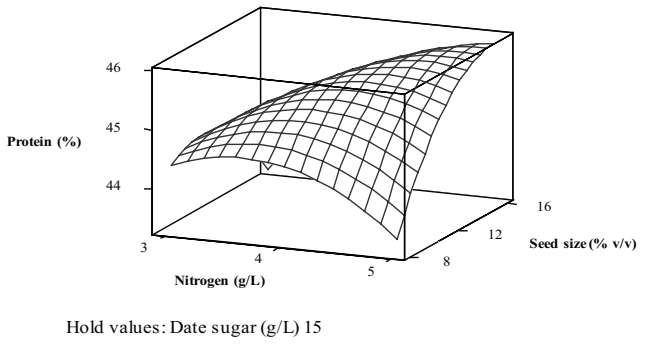


Figure 3: Response surface of interaction effect between nitrogen juice concentration and seed size on mycoprotein production.

Amino acid	Content (% w/w)
Alanine	5.250±1.061
Arginine	6.125±0.187
Aspartic	5.750±0.353
Cystine	3.250±1.061
Glutamic	13.125±0.177
Glycine	4.750±0.353
Histidine	3.000±0.707
Isolucine	4.250±0.353
Leucine	2.750±0.353
Lysine	7.250±1.061
Methionine	3.000±0.707
Phenylalanine	4.500±0.707
Proline	2.250±0.353
Serine	5.750±0.353
Theronine	3.500±0.707
Tyrosine	4.500±0.707
Valine	5.000±0.707

Table 5: Amino acid composition of fungal biomass produced by *F. venenatum*.

Fatty acid	Content (% w/w)
Myristic (C14:0)	0.384±0.047
Pentadecanoic (C15:0)	0.152±0.014
Palmitic (C16:0)	14.253±0.945
Palmitoleic (C16:1)	0.450±0.007
Margaric (C17:0)	0.205±0.002
Heptadec-9enoic (C17:1)	0.121±0.019
Stearic (C18:0)	6.967±0.146
Elaideic (C18:1t)	0.079±0.007
Oleic (C18:1)	18.439±0.653
Linelaideic (C18:2t)	0.076±0.002
Linoleic (C18:2)	30.859±0.638
γ-Linolenic (C18:3)	0.328±0.002
α-Linolenic (C18:3)	25.180±0.748
Arashidic (C20:0)	0.519±0.048
Gadoleic (C20:0)	0.194±0.003
Behenic (C22:0)	0.421±0.034
Erucic (C22:1)	0.142±0.017
Linoceric (C24:0)	0.739±0.69

Table 6: Fatty acid composition of fungal biomass produced by *F. venenatum*.

Conclusions

In this study, process variables in surface culture of *F. venenatum* for protein production from date juice were investigated by response surface methodology. Optimization of variables of date juice and nitrogen source concentrations as well as seed size on mycoprotein production was done by applying FCCD. The protein content of biomass was obtained 46.48% under optimum conditions (date juice 20 g/L, $(NH_4)H_2PO_4$ 4.48 g/L and seed size 12.97% v/v). The results suggest that date juice can be used to produce fungal protein without substantial modification. The scale up of mycoprotein production on modified vogel medium based on date juice is the future trend of this research.

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