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Production of Mycoprotein by *Fusarium venenatum* Growth on Modified Vogel Medium

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Fusarium venenatum (ATCC 20334) was used for fungal protein production. Date sugar was selected as a carbon source. An 8 run Plackett-Burman design was used to study the effect of 7 variables *i.e.*, medium components, temperature, incubation time and inoculum condition on biomass and protein production. The most significant parameters were the seed size (10 % v/v) and carbon source concentration (10 g/L), followed by the temperature (28 ºC) and nitrogen source content (3.5 g/L). In the best condition 47.34 % (w/w) crude protein in the dry product was obtained.

Key Words: *Fusarium venenatum*, Mycoprotein, Plackett-Burman design.

INTRODUCTION

Mycoprotein is the generic name given to the ribonucleic acid-reduced biomass comprising the hyphae (cells) of the organism culture PTA 2684 grown under aerobic condition in a continuous fermentation process[1]. The animal protein sources would be insufficient to meet man's requirements for protein has led to the search for suitable, high-protein, microbial substitutes. Initially this research focused on the use of various yeasts, later, this interest spread to the use of both bacteria and filamentous fungi[2]. A fungus rather than a yeast or bacterium was chosen for the project because (a) of the long history of man using fungi as food (b) it is possible to formulate food products from filamentous fungi which have the appropriate smell, taste and texture and (c) it is relatively easy to harvest fungal mycelia from culture broths[3]. Rank Hovis McDougall (RHM) Company in England decided to produce its new protein-rich food from the filamentous fungus. Strain ATCC PTA-2684 *F. venenatum* was selected as the best organism for myco-protein production. The nutritional value of mycoprotein has been demonstrated the nutritional value of mycoprotein. After receiving MFAFF approval, mycoprotein was sold under the trade name Quorn in the United Kingdom[4]. Quorn is the registered trade name of the product and mycoprotein, a term coined by the foods standards committee is the generic name of the food[5].

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Mycoprotein is a good source of protein and also fiber. The composition of the fiber is about one-third chitin and two-thirds β-1, 3 and 1, 6 glucans. The fat content of the harvested material is typically 2-3.5 % and the fatty acid composition is much more like vegetable than animal fat (polyunsaturated/saturated ratio 3.5:1, tri and diglycerides 65 %, total lipid sterols and unsaponified lipids 5 % and phospholipids 30 %)[1].

Mycoprotein may function as a prebiotic in the lower gut. Turnbull and Ward[6] investigated the effect of mycoprotein consumption on acute glycemia and insulinemia in normal healthy individuals showed that glycemia was reduced post-meal compared to the control and was statistically significant at 1 h (13 % decrease). Insulinemia was also reduced post-meal compared to the control and was statistically significant at 0.5 and 1.0 min (19 and 36 % reduction, respectively). The authors concluded that mycoprotein could be of benefit in the dietary treatment of diabetes[6].

In the last decades, statistical experimental methods have been applied to media optimization for industrial purposes. These designs include blocking and factorial experiments for determining the path of steepest ascent, in order to identify the effect of individual factors and to approach the neighborhood of the optimum response[7]. The Plackett-Burman design (PBD) has been frequently used for screening process variables that make the greatest impact on a process[8]. It is a set of small and efficient experimental design, which is very powerful, widely applicable and especially well suited for biotechnology research and development[9]. For mycoprotein production the defined medium of Vogel was used, with glucose as the carbon source[10]. In our previous study *F. oxysporum* (PTCC 5115) was used to investigate mycoprotein production. Conditions for the optimum production of mycoprotein were determined and heat treatment was used for the reduction of RNA levels in the biomass. The results of our previous experience are now compared with *F. venenatum*[11].

The aim of this research was mycoprotein production with sugar date as the carbon source instead of glucose in Vogel medium[10] using a culture of *Fusarium venenatum* (ATCC 20334). In this study Plackett-Burman design was used to assess the relative importance of medium components (carbon, nitrogen and phosphorus source concentration) and process variables (temperature, time, seed age and seed size) on mycoprotein production.

EXPERIMENTAL

Microorganism, media, inoculum and culture condition: *F. venenatum* ATCC 20334 was used throughout this investigation. Strain was maintained at 4 °C on agar-solidified Vogel slants. The defined medium of Vogel[10] was used with glucose as the carbon source for preculture cultivation. Vogel medium consisted of: 10 g glucose, 2.6 g $Na_3C_6H_5O_7 \cdot 2H_2O$, 2.52 g KNO_3, 2.88 g $(NH_4)H_2PO_4$, 1.6 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g, $CaCl_2 \cdot 2H_2O$, 2.5 mL of biotin solution and 5 mL of trace elements per liter. The trace elements solution consisted of 0.1 g citric acid, 0.1 g

$ZnSO_4 \cdot 7H_2O$, 0.02 g $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$, 5 mg $CuSO_4 \cdot 5H_2O$, 1 mg $MnSO_4 \cdot H_2O$, 1 mg H_3BO_3, 1 mg $Na_2MoO_4 \cdot 2H_2O$ per 100 mL. The pH of the medium was to 5.8.

Inoculum was prepared in 250 conical flasks containing 50 mL Vogel medium. Flasks were inoculated from slants and incubated on a rotary shaker (25 °C, 200 rpm, 48 & 72 h). Date sugar which was purchased from Dombaz company (Iran) containing 76.28 % solids (fructose 37.4 %, glucose 34.1 %, sucrose 0.08 % and ash 7 %) and 23 % water. Production medium was contained of date sugar as a carbon source; other medium components were the same as in seed medium described earlier.

Fermentation was conducted in 500 mL flasks each containing 100 mL of production medium, inoculated with (5 or 10 % v/v depend to trials combination of 8-run PBD) of fungal suspension and incubated for surface culture on various temperatures.

Analytical methods: Procedures of the association of official analytical chemists (AOAC)[12] were used for the analysis of dry weight and protein. Crude protein was measured using the Kjeldahl technique. The crude protein content of the biomass was calculated as 6.25 times the total nitrogen and protein content (w/w), represented by g protein per 100 g total dry solids.

Experimental design

A well defined statistical experimental design is considered to be necessary for optimization of a fermentation process, since it would be possible to get more information through conducting fewer measurements during the process. Plackett-Burman design is one of the highly fractional designs which allows for the study of k = (N-1)/(L-1) factors, each with L levels in N experimental trials. The usefulness of the design lies in the fact that in determining the effects of one variable, the net effects of changing other variables cancel out so that the effect of each variable on the system can be independently determined.

Choice of these factors was based on previous experience (unpublished data) for growing mycoprotein producing fungi and selection of settings reflects a wide but reasonable numerical range. Some changes in the response (protein yield) were also expected for each factor over the selected range.

As it is known, in the Plackett-Burman design only 2 levels can be considered for each factor. Table-1 shows the factors that include some medium components (*i.e.*, carbon, nitrogen and phosphorus source concentration), environmental factors (*i.e.*, temperature and time) and inoculum condition (seed age and seed size). The corresponding levels in this table were chosen on the basis of the preliminary tests. Table-2 shows selected experimental factors and a Plackett-Burman design for conducting eight experimental trials. All the trials were done in triplicate. The elements, + (high level) and - (low level) represent the 2 different levels of the independent factors examined.

TABLE-1
HIGHER AND LOWER LEVELS ASSAYED FOR THE
7 VARIABLES IN PLACKETT-BURMAN DESIGN

	Variables	Low level (-)	High level (+)
A	Date sugar (g/L)	7	14
B	$(NH_4)\ H_2PO_4$ (g/L)	2.88	3.5
C	KH_2PO_4 (g/L)	1.6	2.5
D	Temperature (ºC)	25	28
E	Time (h)	72	96
F	Seed age (h)	48	72
G	Seed size (% v/v)	5	10

RESULTS AND DISCUSSION

In this work, 7 factors were considered: carbon, nitrogen and phosphorus source concentration, temperature, time, seed age and seed size. Minitab 11 software was used to reconfirm of our experimental matrix (24 run); three replicates were used and experiments were randomized. The response signal was the protein yield. The basic equation set up for manual computation of the statistical design was as follows. The coefficients for the eleven variables were determined by:

$$A_i = (1/N)\sum_{0}^{n} X_i \cdot K_i$$

where A_i = coefficient values, X_i = experimental yield, K_i = coded value of each variable corresponding to the respective experimental yield X_i and N = number of experiments. Table-2 gives a comparison of the experimentally determined citric acid production yield and productivity to those predicted by solving the above equation, where predicted yield is given by:

$$Y_i = \sum_{i=0}^{N} A_i \cdot K_i$$

for i = 0, a dummy level of +1 was used and the coefficient obtained was called A_0. The standard error was determined as the sum of the squares of the difference between the experimental and predicted yield for each run. The estimated error is given by:

$$S_b = \sqrt{S_e^2/N}$$

The student's t-test was performed to determine the significance of each variable employed (t-value = coefficient/S_b). Since the experiments were designed to evaluate the relative effect of each variable on response, a significant level of 0.30 is acceptable[13]. However, variables with a high significant effect (at $p < 0.1$ and $p < 0.15$) have been emphasized by underline.

Statistical calculations for Plackett-Burman design of mycoprotein production from date sugar is summarized in Table-3.

TABLE-2
EXPERIMENTAL PLACKETT-BURMAN DESIGN MATRIX AND PROTEIN YIELD OBTAINED[a]

Run	A	B	C	D	E	F	G	Protein yield (g/L)
1	1	-1	-1	1	-1	1	1	47.34 ± 0.55
2	1	1	-1	-1	1	-1	1	46.76 ± 0.66
3	1	1	1	-1	-1	1	-1	40.30 ± 2.25
4	-1	1	1	1	-1	-1	1	43.98 ± 0.57
5	1	-1	1	1	1	-1	-1	42.26 ± 5.58
6	-1	1	-1	1	1	1	-1	40.45 ± 6.71
7	-1	-1	1	-1	1	1	1	39.34 ± 1.90
8	-1	-1	-1	-1	-1	-1	-1	36.39 ± 2.71

[a]All data are the average of 3 replications with standard deviations of the means.

TABLE-3
STATISTICAL RESULTS FOR PLACKETT-BURMAN DESIGN[a]

Factors	Effect	Coefficient	t-value	p-value
A	4.1233	2.0617	6.24	0.000
B	1.5385	0.7692	2.33	0.033
C	-1.2650	-0.6325	-1.92	0.074
D	2.8117	1.4058	4.26	0.001
E	0.2000	0.1000	0.30	0.766
F	-0.9433	-0.2467	-0.75	0.466
G	4.5083	2.2542	6.83	0.000

[a]Tabulated t-value for degree of freedom 6 and $\alpha = 0.05$ is 2.447.

The confidence levels above 95 % (p value < 0.05) were accepted as significant variables. Table-3 shows the statistical results of the different variables. The results show that the carbon and nitrogen sources concentration, seed size and temperature are significant, as their *p*-value were less than 0.05. These factors had positive effects on protein yield. Moreover, the effect of each variable is shown in Fig. 1 as

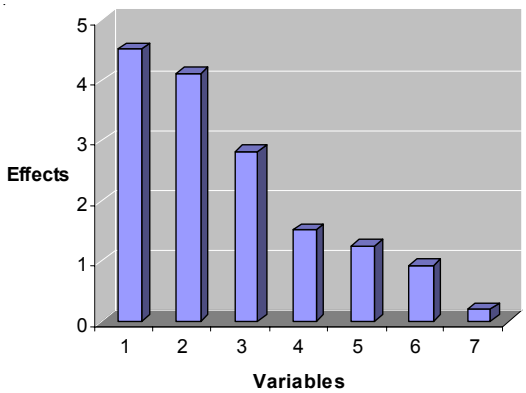

Fig. 1. Results obtained in Plackett-Burman design (1: seed size, 2: carbon source concentration, 3: temperature, 4: nitrogen source concentration, 5: phosphorus source concentration, 6: seed age, 7: time)

it can be seen, the most significant parameters were the seed size and the carbon source concentration, followed by the temperature and the nitrogen source concentration. The other parameters were more or less equally relevant.

The optimum parameters were obtained as date sugar 14 g/L (10 g/L glucose), $(NH_4)H_2PO_4$ 3.5 g/L, KH_2PO_4 1.6 g/L, temperature 28 °C, time 72 h, seed age 48 h and seed size 10 % v/v. Under the best condition 47.34 % crude protein in the dry product was obtained.

In this work a Plackett-Burman design has been used. This is a special design to estimate only the significant parameters. After identification of significant variables, a central composite design will be used to optimize the levels of these variables.

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