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Screening of Nutrient Parameters for Lovastatin Production by *Monascus purpureus* MTCC 369 under Submerged Fermentation Using Plackett-Burman Design

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Abstract: Lovastatin, an HMG-CoA reductase inhibitor can be produced by *Monascus purpureus*. Plackett-Burman experimental design was used to identify important nutrients useful for the production of lovastatin by *Monascus purpureus* MTCC 369 under submerged fermentation. Nine nutrient parameters such as dextrose, peptone, NH_4Cl, yeast extract, KH_2PO_4, $FeSO_4.7H_2O$, $MgSO_4.7H_2O$, $MnSO_4.H_2O$ and $CaCl_2.2H_2O$ were screened in twelve experiments as per the design. $MnSO_4.H_2O$ and dextrose had contributed to a large extent, yeast extract, $FeSO_4.7H_2O$ and $CaCl_2.2H_2O$ had little impact, while, NH_4Cl, KH_2PO_4 and peptone contributes moderately in production of lovastatin.

Key words: Lovastatin, Plackett-Burman design, *Monascus purpureus*, submerged fermentation

INTRODUCTION

Cholesterol is an important component of the cell membrane. About two-third of the total body cholesterol is synthesized in body and remaining acquired from diet (Alberts *et al*., 1980). Amount of cholesterol should be well maintained in body. High cholesterol level leads to formation of atherosclerotic plaques, which further contributes to the development of coronary heart diseases (CHD) (Endo, 1985; Tobert, 2003). Thus, regulation of cholesterol biosynthesis is beneficial to control CHD. Statins are widely used to reduce elevated blood cholesterol. The mechanism involved in the hypocholesterolemic activity of statins is based on the competitive inhibition of the rate-limiting enzyme of cholesterol biosynthesis, 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase. Inhibition takes place due to structural homology between the biologically active form of statins, β-hydroxy acid and HMG-CoA, which is an intermediate of cholesterol biosynthetic pathway (Manzoni and Rollini, 2002).

Among the statins, mevastatin was the first statin investigated as a fungal secondary metabolite (Chakravarti and Sahai, 2004), later followed by lovastatin (Demain, 1999). Lovastatin, more active methylated form of mevastatin (Endo, 1979), was the first statin approved by US FDA USA in 1987 as a hypercholesterolemic drug (Tobert, 2003).

Numerous fungi namely, *Monascus ruber* (Endo, 1979) *M. purpureus* (Su *et al*., 2003; Wang *et al*., 2003) *M. paxi* (Manzoni and Rollini, 2002), *M. anka* (Su *et al*., 2003), *Aspergillus terreus* (Alberts *et al*., 1980; Hajjaj *et al*., 2001), *Aspergillus flavipes* (Valera *et al*., 2005) *A. fischeri*, *A. flavus*, *A. umbrosus*, *A. parasiticus*, *Accremonium chrysogenum*, *Penicillium funiculosum*, *Trichoderma viridae*, *T. longibrachiatum* (Samiee *et al*., 2003) have been reported for lovastatin production.

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Designing a fermentation medium is a critical and important process as the medium composition can significantly affect the product yield (Kennedy and Krouse, 1999). An optimally balanced culture medium was mandatory for maximal production of the secondary metabolites. Important medium variables are screened by Plackett-Burman experimental design (Plackett and Burman, 1946). It is partial factorial design, where large numbers of independent variables (N) are studied in small number of experiments (N+1) (Naveena et *al*., 2005).

In the present study we report selection of nutrient constituents using Plackett-Burman experimental design for the production of lovastatin by *Monascus purpureus* MTCC 369 in submerged fermentation.

MATERIALS AND METHODS

Microorganism

Cultures of *M. purpureus* MTCC 369 obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India. It is maintained on slants of Potato-Dextrose Agar (PDA) medium at 4°C and sub cultured every 30 days.

Preparation of Seed Culture

Spore suspension of *M. purpureus* MTCC 369 was prepared from actively growing slants in sterile water and diluted to a concentration 5.7×10^3. Fifteen percent spore suspension was inoculated to conical flasks containing the basal medium (100 g dextrose, 10 g peptone, 2 g KNO_3, 2 g $NH_4H_2PO_4$, 0.5 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2$ in 1000 mL distilled water; adjusted to pH 6.0). These cultures were incubated at 30°C for 48 h in a shaker incubator at 110 rpm (Su *et al*., 2003).

Plackett-Burman Experimental Design

Dextrose, peptone, NH_4Cl, yeast extract, KH_2PO_4, $FeSO_4.7H_2O$, $MgSO_4.7H_2O$, $MnSO_4.H_2O$ and $CaCl_2.2H_2O$ were the nine medium constituents selected for study. The Plackett-Burman experimental design (Plackett and Burman, 1946) for eleven variables: nine nutritional components (independent variables) and two dummy variables (Table 2) were used to evaluate the relative importance of various nutrients for lovastatin production in submerged culture. In Table 2, each row represents an experiment and each column represents a different variable. For each nutrient variable two different concentrations high (+) and a low (-) was tested (Table 1).

Submerged Fermentation

All experiments have been carried out in duplicates in 250 mL Erlenmeyer flasks containing 50 mL media as per experimental designs. pH of the broths adjusted to 6.0 with 0.1M HCl or NaOH. These flasks autoclaved at 15 psi and 121°C for 15 min. Then each flask inoculated with 10% seed culture and incubated at 30°C for 14 days on a rotary shaker at 110 rpm (Su *et al*., 2003).

Lovastatin Extraction

Fermented medium was sonicated, adjusted to pH 3.0 using 2N H_3PO_4 and extracted with equal quantity of ethyl acetate. The mixtures were centrifuged at 3000 g for 8 min, 1 mL supernatant was collected and lactonized with 10 mL of 1% trifluoroacetic acid (Su *et al*., 2003). The resultant was concentrated, diluted to appropriate concentration with acetonitrile and filtered through 0.45 μm filter for HPLC analysis.

Lovastatin Estimation

Procedure given by Samiee *et al*. (2003) for HPLC analysis was slightly modified. Lovastatin estimated by HPLC (SHIMADZU, Japan) using 250×4.6 mm ID Lichrosper® 100 C_{18} column of

Table 1: Concentrations of variables at different levels in Plackett-Burman design for lovastatin production in submerged culture

Designation	Variable	Low level (-) (g L^{-1})	High level (+) (g L^{-1})
X_1	Dextrose	40	80
X_2	Peptone	10	40
X_3	NH_4Cl	2	8
X_4	Yeast extract	2	8
X_5	KH_2PO_4	2	8
X_6	Dummy 1	-	-
X_7	$FeSO_4.7H_2O$	0.1	0.9
X_8	$MgSO_4.7H_2O$	0.1	0.9
X_9	$MnSO_4.H_2O$	0	0.5
X_{10}	Dummy 2	-	-
X_{11}	$CaCl_2.2H_2O$	0	0.6

5 μm particle size and 20 μL loop injector. Acetonitrile: Water acidified to the concentration 0.1% with ortho-phosphoric acid (65:35 v/v), was used as mobile phase. Flow rate of mobile phase maintained at 1.5 mL min^{-1} and detection was carried out by UV-detector at 235 nm (Samiee *et al*., 2003).

Data Analysis

The effect of each variable was determined with the following equation (Stanbury *et al*., 1997).

$$E_{xi} = 2 (\sum H_{xi} - \sum L_{xi})/N$$

Where, E_{xi} is the concentration effect of the tested variable, H_{xi} and L_{xi} are the concentrations of lovastatin at high level and low level of the same variable and N is the number of trials. When the sign is positive, the influence of variable upon lovastatin production is greater at high concentration and when the negative, the influence of variable is greater at a low concentration.

Mean square of each variable (the variance of effect) was calculated as follows (Stanbury *et al*., 1997):

$$V_{xi} = (\sum H_{xi} - \sum L_{xi})^2/N$$

Where, V_{xi} is mean square of variable.

The experimental error was calculated by averaging the mean squares of the dummy variables (Stanbury *et al*., 1997).

$$R = \sum V_{xd}/n$$

Where, R is the experimental error (mean square for error), V_{xd} is mean square of dummy variable and n is number of dummy variables (Chakravarti and Sahai, 2004).

Factor showing larger effects were identified using F-test (Stanbury *et al*., 1997).

$$F = V_{xi}/R$$

RESULTS

Screening of nutrients by Plackett-Burman experimental design for lovastatin production using *M. purpureus* MTCC 369 were carried out in this study, the goal was to select the important nutritional variables. In this experiment different compounds were tested for their nutritional ability in lovastatin production. Maximum lovastatin production was found in experimental trial 6, whereas, minimum in 7 (Table 2) under submerged fermentation using *M. purpureus* MTCC 369. Effect of

Table 2: Plackett-Burman experimental design of 12 trials for eleven variables (+ High level, - Low level) along with observed concentration of lovastatin in fermented broth

Trial	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	Lovastatin (mg L^{-1})
1	+	+	-	+	+	+	-	-	-	+	-	0.0695
2	-	+	+	-	+	+	+	-	-	-	+	0.1721
3	+	-	+	+	-	+	+	+	-	-	-	0.1438
4	-	+	-	+	+	-	+	+	+	-	-	0.2360
5	-	-	+	-	+	+	-	+	+	+	-	0.2691
6	-	-	-	+	-	+	+	-	+	+	+	0.3180
7	+	-	-	-	+	-	+	+	-	+	+	0.0318
8	+	+	-	-	-	+	-	+	+	-	+	0.1040
9	+	+	+	-	-	-	+	-	+	+	-	0.2435
10	-	+	+	+	-	-	-	+	-	+	+	0.1689
11	+	-	+	+	+	-	-	-	+	-	+	0.2112
12	-	-	-	-	-	-	-	-	-	-	-	0.2018

Table 3: Influence of medium variables on lovastatin production

Designation	Variable	∑H	∑L	Mean square	Effect	F-value
X_1	Dextrose	0.804	1.366	0.02630	-0.094	500.95
X_2	Peptone	0.995	1.176	0.00270	-0.030	51.43
X_3	NH_4Cl	1.209	0.962	0.00510	0.041	97.14
X_4	Yeast extract	1.147	1.023	0.00130	0.021	24.57
X_5	KH_2PO_4	0.990	1.181	0.00300	-0.032	57.71
X_6	Dummy 1	1.077	1.093	0.00002	-0.003	-
X_7	$FeSO_4.7H_2O$	1.145	1.025	0.00120	0.020	23.05
X_8	$MgSO_4.7H_2O$	0.954	1.216	0.00570	-0.044	109.05
X_9	$MnSO_4.H_2O$	1.382	0.788	0.02940	0.099	560.76
X_{10}	Dummy 2	1.101	1.069	0.00008	0.005	-
X_{11}	$CaCl_2.2H_2O$	1.007	1.164	0.00210	-0.026	39.20

dummy variable 1 and 2 were close to zero (Table 3), indicates successful experimental work. Experimental error was calculated and found to be 0.00005. Among the nine nutrient components used in study, $MnSO_4.H_2O$ and dextrose had contributed to a large extent for lovastatin production. Yeast extract, $FeSO_4.7H_2O$ and $CaCl_2.2H_2O$ had little impact, while, NH_4Cl, KH_2PO_4 and peptone contributes moderately in production of lovastatin. NH_4Cl and $MnSO_4.H_2O$ were influences the production in their higher concentration, where as dextrose, $MgSO_4.7H_2O$, KH_2PO_4 and peptone were effective in lower levels.

DISCUSSION

It was found that dextrose is useful as a carbon source; it has high contribution and effective in lesser amount as dextrose was a significant limiting factor influencing the regulation of lovastatin biosynthesis via growth (Miyake *et al*., 2006). Ammonium chloride was found to be better nitrogen source than yeast extract and peptone for lovastatin production by *Monascus purpureus* MTCC 369. This is one reason that the growth of *Monascus purpureus* (biomass) was largely depending on type of nitrogen source. Moreover, a suitable concentration of ammonium chloride was important for higher lovastatin production, lower biomass at lower ammonium chloride concentration resulted less production of lovastatin (Miyake *et al*., 2006). In fungal nutrition magnesium and calcium are noted as macronutrients and manganese, iron, copper and zinc as micronutrients but in case of lovastatin production by *Monascus purpureus*, manganese contribution was higher then calcium, iron and magnesium. This may due to manganese acting, as cofactor for different enzymes require for lovastatin production (Yu *et al*., 1997).

Designing the medium is an open ended, time-consuming and laborious process involving large number of experiments. The Plackett-Burman experimental design is the preliminary technique for rapid illustration of the effects of various medium constituents. It tests each variable at two levels only; hence it can not give exact idea regarding the optimum level of constituent required in the medium.

Therefore, further optimization of selected nutrients such dextrose, ammonium chloride, $MnSO_4.H_2O$, $MgSO_4.7H_2O$ and KH_2PO_4 for lovastatin production by *Monascus purpureus* MTCC 369 is necessary.

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