Screening and Optimization of Carbon and Nitrogen Source for Increasing Lovastatin Production by Submerge Fermentation Using Aspergillus Sp.

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Abstract: In this study the production of lovastatin by submerged fermentation using Aspergillus sp. wascarried out in shake flask. The objective of this study was to improve lovastatin production by screening and optimization of carbon and nitrogen sources. Carbon sources such as glucose, lactose, starch, and molasses alone and in combination and different nitrogen sources (yeast extract, soybean meal, ammonium nitrate, corn steep liquor, sodium glutamate) were evaluated in terms of lovastatin production. Other medium constituents and culture conditions were held constant while only carbon and nitrogen sources were changed in separate experiments. The shake flask study was carried out for a period of 168 h. The highest lovastatin production of 541 mg/l was obtained at 144 h, using the medium containing glucose and lactose as carbon source and Na-glutamate as a nitrogen source. A response surface analysis was performed based on the central composite design to determine the C: N ratio optimal for lovastatin production. A cubic model was developed to elucidate the combined effect of glucose, lactose and sodium glutamate concentrations. The optimized value obtained by the statistical analysis showed that glucose at 22.78 gm/l, lactose 26 gm/l and 14.48 gm/l Na-glutamate was optimum for the maximum lovastatin production after 144 h of fermentation.

IndexTerms **- Lovastatin; Carbon and Nitrogen Sources; Statistical design; Fermentation; Aspergillus sp.**

I. INTRODUCTION

Lovastatin ($C_{24}H_{36}O_5$) is a potent cholesterol lowering drug, which is used as an important first line therapy in a majority of the patients suffering with high blood cholesterol. Besides dietary sources, cholesterol is also synthesized by the body chiefly by the liver. Lovastatin reduces total and low density lipoprotein (LDL) cholesterol levels by competitively inhibiting 3-hydroxy-3-methylglutaryl-conenzyme A (HMG -Co A) reductase enzyme and thus blocking endogenous formation of cholesterol. HMG - Co A reductase enzymecatalyzes the conversion of HMG-Co A to mevalonate, which is the rate limiting step in the biosynthesis of cholesterol. It also improves blood flow by restoring the function of the inner lining of arteries affected by atherosclerosis. Lovastatin can be produced by secondary metabolism of various fungi including Penicillium sp. [5], Monascus sp. [9] Aspergillus terreus [1] through the polyketide biosynthetic pathway.

The formulation of fermentation media becomes important, especially when secondary metabolites are produced [12]. Carbon and nitrogen sources are one of the major nutrients of culture medium, which play an important role infermentation productivity because these are directly associated with the biomass formation and metabolite production. Lovastatin production by A. terreus was influenced by the type of carbon and nitrogen sources and its ratio in the medium [2]. The genes and enzymes involved in lovastatin biosyntheses by A. terreus has been identified and characterized [8, 10]. Now it is known that both carbon and nitrogen sources regulate polyketide biosynthesis by exerting complex regulation on related gene expression and enzyme activity [11].

As with any process development, the culture mediumplays a significant role on the yield of product and its rate of production. Selection of these carbon and nitrogen sources and optimization of its composition is therefore important in establishing a fermentation process for production of lovastatin. Various statistical experimental design strategies have been used to optimize fermentation media for lovastatin production by different species [4, 3, 13). Since, secondarymetabolite production is directly linked to the type and concentration of carbon and nitrogen sources, different carbon and nitrogen sources were evaluated in terms of lovastatin production by Aspergillus sp. After determining the carbon and nitrogen sources that resulted in satisfactory lovastatin production, a statistical experimental design method was used to optimize the carbon: nitrogen ratio to develop a defined medium for improving lovastatin production. The information obtained here is considered fundamental because it gives pellet morphology of Aspergillus sp. which in turn reduces mass transfer limitations to a significant degree and hence makes the process suitable for operation at a larger scale.

II. MATERIALS AND METHODS

2.1. Microorganism and Inoculation Preparation

Aspergillus sp. was used in the present study. It was maintained on slants of potato dextrose agar medium, stored at 4°C and was sub cultured at every 15 days.Spore suspension of Aspergillus sp. was prepared by adding 10 ml of 0.1% solution of tween 80 in actively growing (6-8 days old) slants. The surface was scratched with an inoculation needle and mixed thoroughly by vortexingto suspend the spores. The number of spores in the spore suspension was counted using a hemocytometer and the suspension was diluted to adjust the spore concentration to 1×108 spores/ml. A spore suspension of thisconcentration was used as inoculum throughout the study.

2.2 Experiments on carbon sources

All the experiments were carried out in 250 ml Erlenmeyer flask containing 50 ml medium. Effects of different carbon sources on submerged culture of Aspergillus sp. was evaluated using 20 gl-1 of one of the following carbon sources, i.e. glucose, lactose, molasses, starch, alone and in combination. The other components used in the medium included Na-glutamate: 12.5 g l-1, KH2PO4: 5 gl-1, K2 HPO4: 5 gl-1, MgSO4.7 H2O: 0.1 gl-1 CaCl2.2H2O: 20 mg l-1, CuCl2.2H2O: 5 mgl-1, H3BO3: 11 mgl-1,(NH4)6Mo7O24.4 H2O: 5 mgl-1 and adjusted to pH 6.5. Flasks containing 50 ml medium were inoculated with 2.5 ml of spore suspension and incubated at 30ºC for 7 days in a rotary shaker at 150 rpm.

2.3 Experiments on nitrogen sources

The experiments were carried out similarly as explained in section 2.2 except that only glucose (20 gl-1) and lactose (20 gl-1) were used incombination as carbon sources; different nitrogen sources such as Na-glutamate, yeast extract, urea, ammonium nitrate, soybean meal, corn steep liquor (12.5 gl-1) were used with these two carbon sources in separateexperiments. The other medium components and culture conditions were held constant.

2.4 Experimental design

The central composite design (CCD) was employed to determine the optimum levels of carbon and nitrogen sources for lovastatin production. An experimental design of 20 experiments was formulated according to surface response methodology using Design Expert 5.9 software (Statease, USA). The various levels of carbon and nitrogen sources used for the experimental design have been summarized in Table 1. The individual experiments were carried out for seven days.

Table 1 Actual and coded values of the variables used in the experiment

Response Variable	Coded level of variable				
	-α	-1	0	+1	+α
Glucose (gm/l)	3.18	10.0	20.0	30	36.82
Lactose (gm/l)	3.18	10.0	20.0	30	36.82
Na-Glutamate (gm/l)	0.0	5.0	12.5	20	25.11

The relative effects of these three variables on lovastatin production were identified from contour plots and three dimensional graphs. An optimum value of the factors for maximum lovastatin production was determined by point prediction tool of the software.

2.5 Analytical procedures

2.5.1 Determination of dry cell weight (DCW)

The dry cell weight was estimated by filtering a known volume of broth through pre weighed Whatman filter paper (Whatmann No. 1) and drying the cells at 70ºC for 8 hrs.

2.5.2 Lovastatin Extraction and Estimation

The culture broth obtained through shake flask was extracted by adding equal volume of methanol and pH was adjusted to 7.7 with the help of 2N NaOH in 250 ml flask. The mixture was kept in constantly agitated rotary shaker for 2 h at 30○C and 200 rpm. After 2 h, the mixture was filtered through 0.45 µm membrane filter [14]. Lovastatin was estimated by HPLC using Agilent 1100 series Nova PakTM C18 column (250 mm length X 4.6 mm ID). A modified Friedrich's method [6] was followed for lovastatin estimation. A mixture of 0.02 M phosphate buffer (pH 7.7) and acetonitrile in ratio of 65:35 (v/v) was used as mobile phase. The flow rate of mobile phase was maintained at 1.0 ml/min and detection was carried out by a UV detector at 238 nm.

3. RESULT

3.1 Effect of various carbon source on biomass and lovastatin production.

A comparative study was carried out to identify a suitable carbon source for lovastatin production by submerged cultivation of *Aspergillus* sp. by growing in different carbon sources i.e. glucose, lactose, molasses, starch alone and in combination The time profiles of cell growth and lovastatin production under various carbon sources were compared in Fig. 1a and 1b. It was observed that *Aspergillus* sp. Supported growth on all carbon sources. It was also observed that lovastatin production was limited in presence of single carbon source but increased when a combination of carbon sources were used. A maximum dry cell weight and lovastatin production was obtained when both glucose and lactose was used as a carbon source. After 144 h of shake flask cultivation, a biomass concentration of 22.16 g DW $l^{-1}$and lovastatin concentration of 541.74 mg l^{-1} was achieved. The results in Figs 1a and b clearly indicate that a combination ofrapidly and slowly metabolizable carbon source, improves both growth and productivity as compared to the use of individual carbon sources. Glucose is used predominantly during the growth phase while lactose in the stationary phase. A similar

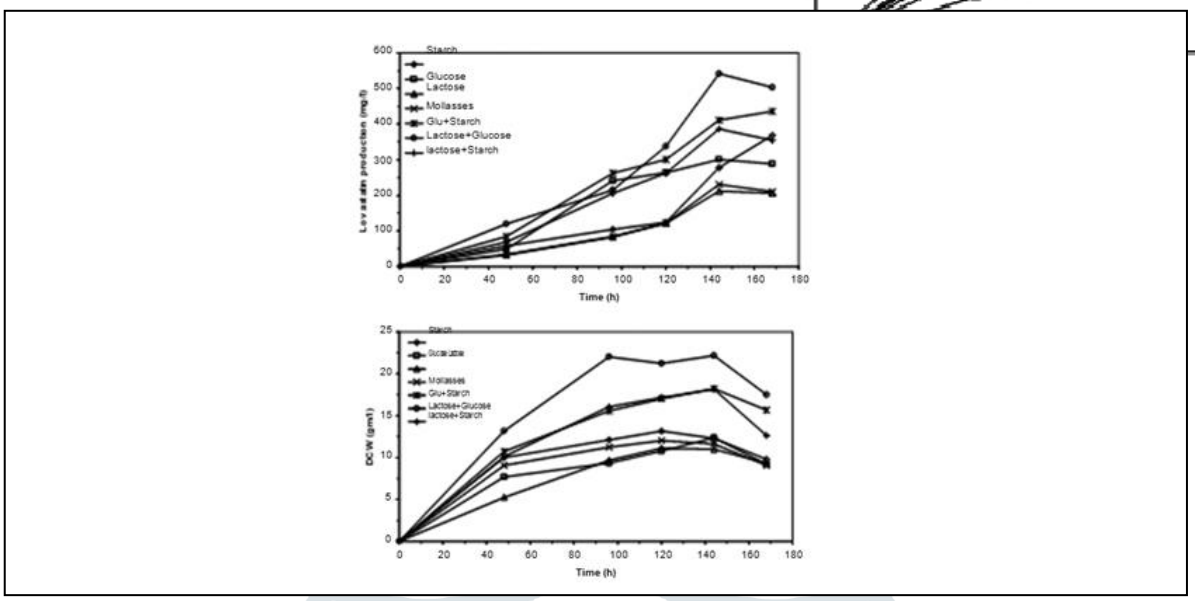

Fig.1a. Influence of various carbon sources on the lovastatin production, Fig. 1b Influence of various carbon sources on the biomass formation

3.2 *Effect of nitrogen source on the growth and lovastatin production*

To increase the lovastatin production further the different nitrogen sources including yeast extract, urea, ammonium nitrate, corn steep liquor, soybean meal, Na-glutamate were evaluated in terms of lovastatin production and dry cell weight. Among all nitrogen sources evaluated, Na-glutamate was found to be most suitable for lovastatin production. Comparative results of all nitrogen sources are presented in fig. 2a and 2b. The maximum lovastatinproduction of 536.48 mg l^{-1} was found after 144 h of shake flask cultivation in the presence of Na- glutamate in the medium. In the presence of Na- glutamate the maximum dry cell weight obtained was 24.32 g l^{-1} after 144 h.

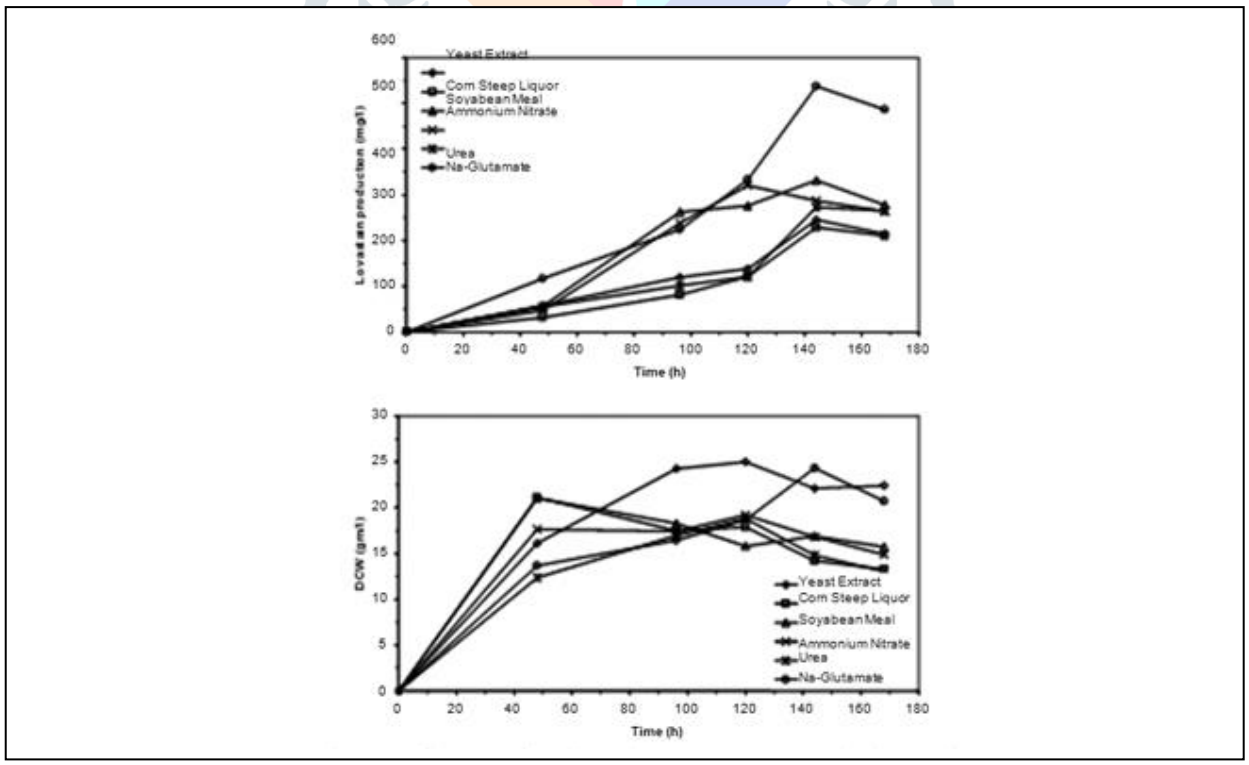

Fig.2a. Influence of various nitrogen sources on the lovastatin production, 2b: Influence of various nitrogen sources on the biomass formation

3.3 *Optimization of carbon and nitrogen concentration in shake flask by central composite design*

From the above study glucose and lactose as a carbon source and Na- glutamate as a nitrogen source are selected for further study. The concentrations of both carbon and nitrogen sources and their ratio in medium play a vital role for optimal metabolite production. The combined effect of the carbon and nitrogen sources on growth and lovastatin production was investigated by using the statistical approach of surface response central composite design (CCD). Table 2 shows the detailed experimental design and result.

Table 2 Experimental design and result

Run	Glucose (g/l)	Lactose (g/l)	Na Glutamate (g/l)	Lovastatin mg/l
1	10.00	30.00	20.00	497.99
2	20.00	3.18	12.50	501.70
3	30.00	10.00	5.00	553.12
4	30.00	30.00	20.00	640.40
5	10.00	30.00	5.00	279.77
6	30.00	10.00	20.00	283.66
7	20.00	20.00	12.50	550.29
8	36.82	20.00	12.50	309.42
9	20.00	20.00	12.50	530.81
10	20.00	36.82	12.50	377.54
11	20.00	20.00	12.50	527.38
12	10.00	10.00	5.00	257.76
13	10.00	10.00	20.00	335.02
14	30.00	30.00	5.00	304.61
15	20.00	20.00	12.50	509.1
16	3.18	20.00	12.50	305.92
17	20.00	20.00	12.50	539.07
18	20.00	20.00	25.11	313.69
19	20.00	20.00	12.50	569.22
20	20.00	20.00	0	0

The lovastatin production was analyzed using the software DESIGN EXPERT 5.0.9 and fitted into cubic model proposes following equation (in the coded factor) for lovastatin production.

$$\text{Lovastatin } (\text{mgl}^{-1}) = 534.19 + 78.95 * A + 88 * B + 18.96 * C + 58.73 * A^2 + 76.12.08 * B + 112.05 * C + 90.59 * AB + 28.64 * AC + 93.28 * BC + 27.55 * A^2 + 40.23 * B^2 + 26.27 * C^2 + 38.04 * A * B * C$$

Where A, B and C represent glucose , lactose and Na-glutamate respectively in g l^{-1}. The ANOVA for the experiments performed has been presented in Table 3. A correlation coefficient of R^2 = 0.9162 (a values of R^2 > 0.75 indicates the fitness of the model) was obtained for lovastatin concentration data, showing that the cubic mdel used for its prediction was satisfactory. The predicted sum of squares (PRESS), which is a measure of how a particular model fits each point in the design was 8.062E + 06 (for lovastatin production). Values of "Prob>F " less than 0.05 indicated that model terms were significant.

Table 3 ANOVA for response surface cubic model obtained for lovastatin production

Term	Lovastatin Production
F -Value	5.05
P> F[a]	0.0285(< 0.05)
DF	13
R^2	0.9162
RMSE	80.30
Dep Mean	409.32
C.V.	19.62
PRESS	8.062E + 06
Adj R^2	0.7347
Adeq Precision[b]	7.997 (Desire > 4)
Model Sum of Squares	4.231E+05
Residual Sum of Squares	38687.36
Model Mean Square	32547.18
Residual Mean Square	6447.89

[a] value of "P>F" less than 0.05 indicate model terms are significant.
[b] The value >4 indicates adequate precision in the model.

Three dimensional response surface graphs were plotted to study the interactions among the various selected factors and to determine their optimum concentrations for attaining maximum yield of lovastatin. The response surfaces obtained for lovastatin have been presented in figs 3a, 3b and 3c. Finally, from a complete analysis of the data, the optimum values of 22.78 gml^{-1} glucose, 14.48 gml^{-1} sodium glutamate and 26 gml^{-1} lactose were determined as the compositon resulting is maximum lovastatin production. The predicted loavstatin for this composition was 581 mgl^{-1}.

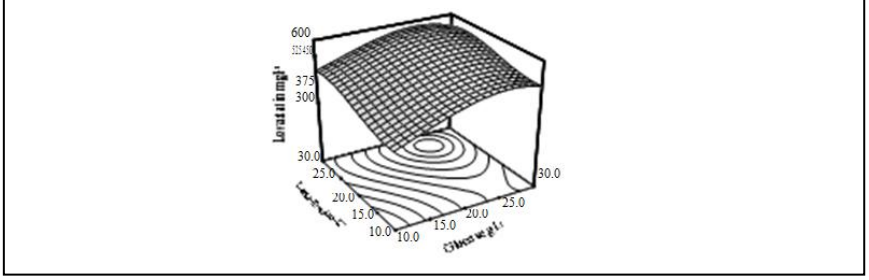

Fig. 3a. Lovastatin production response to variations in the different concentration of the lactose and glucose.

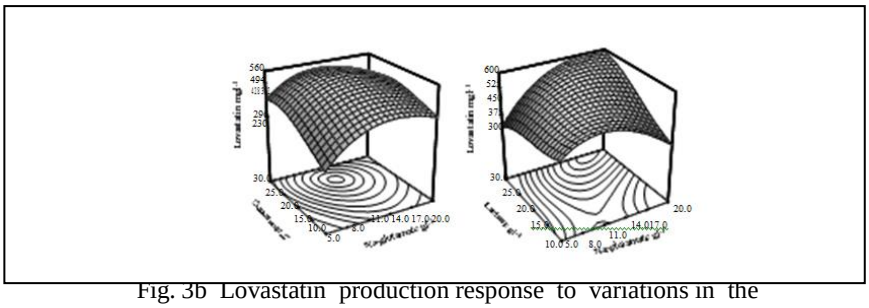

Fig. 3b Lovastatin production response to variations in the different concentration of the glucose and Na-glutamate. Fig. 3c Lovastatin production response to variations in the different concentration of lactose and Na-glutamate

4. CONCLUSION

A fermentation process of submerged cultivation of *Aspergillus* sp. in shake flask for production of a secondary metabolite, lovastatin, was investigated. The effects of different carbon and nitrogen sources and their ratios on lovastatin production were studied to formulate a suitable fermentation medium. A combination of glucose and lactose carbon sources along with Na-glutamate as a nitrogen source was found to be the most suitable for maximizing lovastatin production. A surface response analysis was performed to determine the effect of the different concentration of carbon and nitrogen sources for lovastatin production. It has been found that the 581 mgl^{-1} of lovastatin was achieved by using glucose 22.78 gm^{-1}, lactose 26 gm^{-1} and Na-glutamate 14.48gm^{-1}. This fundamental information obtained in this work is helpful for further development of *Aspergillus* sp. Cultivation process for enhancement of lovastatin production on large scale.

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