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Process Optimization of Mevinolinic Acid by *Aspergillus terreus* through Submerged Fermentation

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ABSTRACT:

Mevinolinic acids are the secondary metabolic compounds produced by some fungal strains and are widely used to reduce elevated levels of cholesterol in blood plasma. Optimizing a fermentation system involves optimizing many variables such as determining the effect of seed quality and media components, and selecting the most appropriate operating conditions. In present study, pilot scale optimization of Mevinolinic acid production through submerged fermentation by *Aspergillus terreus* has been described. Optimization of Mevinolinic acid production was carried out by studying physical parameters such as the effect of incubation temperature, initial pH and nutritional parameters. An incubation temperature of 28ºC and the initial pH of 6.5 were found optimum for Mevinolinic acid production. Highest production was observed when seed transfer volume was observed at 10 % and the D.O. was maintained between 40 -60% throughout the batch. Five different kinds of media (M1-M5) were used in the study out of which the maximum production of Mevinolinic acid was observed in M4 since it had amino acids, essential vitamins and mineral salts which were provided by other components of media such as skim milk, cotton seed meal, soyabean flour, glycerol and other components.

Key words: Aspergillus terreus, Mevinolinic acid, Fungal Morphology, Process Optimization Submerged Fermentation.

1. INTRODUCTION:

Large scale fermentations to grow microbial cells, and intra or extra-molecular components involve growing the appropriate micro-organism on a complex media, normally in submerged fermentation vessels. Medium composition can significantly affect product concentration, yield and volumetric fermentation productivity. Medium cost can substantially affect overall process economics, especially of commodity products. Therefore, designing a fermentation medium is critical when developing an industrial fermentation process. Homogeneous conditions for temperature, pH, dissolved oxygen, substrate, and product concentration have to be maintained in the reactors to ensure consistent processes and products. The bioprocess needs to be controlled

and all available measurements should be logged to enable quality assurance. Safety regulations also have to be followed to prevent accidents and release of toxic products. Therefore, fermentations take time and consequently any experiments are going take time to get the results.

Mevinolinic acid *(*$C_{24}H_{36}O_5$*)* is a potent drug for lowering blood cholesterol. It acts by competitively inhibiting the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA)[1,2] which catalyzes the rate limiting step of cholesterol biosynthesis. Mevinolinic acid also inhibits tumor growth through the inhibition of nonsterol isoprenoid synthesis.[3,4] Mevinolinic acid is produced as a secondary metabolite by a variety of filamentous fungi such as *Penicillium* sp.,[5] *Monascus ruber*[6,7] and *Aspergillus terreus*.[2] Commercial production of Mevinolinic acid is based on *A. terreus* batch fermentation and most of the literature deals with new species.[8–13] *Aspergillus terreus* fermentation processes typically carried out at ~28 ◦C and pH 5.8–7.8.[12] The dissolved oxygen (D.O.) level is quickly maintained to ~40%.[12] A batch fermentation generally runs for 10 days. Pellet growth of *A. terreus* has process for higher yield/ tittered value of Mevinolinic acid after then yielded with filamentous growth.[12]; the productivity of a fermentation process also depends on the culture medium mainly carbon and nitrogen sources play a dominant role in fermentation productivity as these nutrients are directly linked with the formation of biomass and metabolites. Also, the nature and concentration of the carbon source can regulate secondary metabolism through phenomena such as catabolic repression. Biosynthesis of Mevinolinic acid has been found to depend on the carbon and nitrogen sources.[10, 11, 13]

The current study involves the optimization of production medium, seed transfer parameters, and important factors of production parameters such as pH and Dissolve Oxygen (D.O.) for Mevinolinic acid production.

2.FACTORS AFFECTING MEVINOLINIC ACID YIELD:

2.1 Effect of rapidly Metabolizable Substrates and C: N ratio

Uncontrolled filamentous growth occurs when using rapidly metabolized substrates which in turn rapidly increases viscosity of medium & decreases Mevinolinic acid production.

Remedial Measures

(i) Slow use of the carbon source, in particular glycerol, yields a fivefold increase with respect to the initial Mevinolinic acid productivity.

(ii) Combination of lactose and glucose i.e. slowly and rapidly metabolizable sugar under N-limited conditions yields highest Mevinolinic acid titers.

(iii) A cost effective repeated fed-batch process with maltodextrin and corn steep liquor feed as carbon and nitrogen sources, respectively, significantly increases Mevinolinic acid yield.

(iv) The maximum value of the Mevinolinic acid yield on biomass is using the lactose/soybean meal and lactose/yeast extract media.

The metabolic pathways for the synthesis of Mevinolinic acid from carbon are much slower than the pathways that convert carbon to biomass. Therefore, nitrogen limitation (i.e. growth suppression) helps with synthesis of Mevinolinic acid by diverting more carbon to its synthesis. Culture medium should have excess carbon but limiting amounts of nitrogen source for the better productivity. The optimal initial C: N mass ratio for attaining high productivity of Mevinolinic acid is 40 mg/gm.

2.2 Viscosity of Fermentation Broth

The rapid increase in viscosity accompanied by filamentous growth greatly impedes oxygen transfer and this is said to explain the low titers of Mevinolinic acid.

Remedial Measures

(i) Set up a more-efficient impeller with increased hydrodynamic thrust and a reduction of power requirement, 66% of that of the Ruston standard turbine.

(ii) Normally, lower Dissolved Oxygen corresponded to lower agitation. But agitation might also interact with the culturing environments, which in turn affected product formation. Exponential increase in Mevinolinic acid & a better yield is expected when the oxygen carrier like n-dodecane, n-tetradecane and n-hexadecane is added after 24 hrs of fermentation process.

Oxygen transfer, related to high viscosity of the fermentation broth, is a serious limiting factor in Mevinolinic acid productivity. Both a limitation and excess of oxygen reduced Mevinolinic acid titers. With the Dissolved Oxygen controlled at 20%, highly entangled mycelia occurring at earlier stages further clumped to form spherical, compact pellets.

2.3 Age of Spores

Mevinolinic acid production depends on the age of the spores used for inoculation. Mevinolinic acid titer is found to be 16.0 ±2 mg/gm for a spore age of 12 days.

The time to sporulation on surface cultures is sensitive to the light exposure history of the fungus and the spore inoculation concentration level.

2.4 Inhibiting feedback Loop Inhibition

Accumulation of Mevinolinic acid suppresses its own synthesis.

Remedial Measure

(i) Design a cost effective repeated fed-batch process with maltodextrin and corn steep liquor feed as carbon and nitrogen sources, respectively, to favor slow and steady accumulation.

(ii) Try genetic Manipulation to break negative feedback loop.

Negative regulation of secondary metabolite via feedback inhibition loop can only be counteracted by keeping its concentration in fermentation broth below threshold level.

2.5 Pelleted V/s filamentous Growth

Pelleted growth yield higher titers of Mevinolinic acid than obtained with filamentous. The preferred pelleted growth morphology that favors Mevinolinic acid synthesis can be readily obtained and maintained in the bubble column reactor.

Stirred tank fermentation has a substantially lower production of Mevinolinic acid because mechanical agitation damages the fungal pellets. Still if using a Stirred tanks an agitation of 325 rpm to achieve Mevinolinic acid production at 16.0 mg/gm at day 10 is recommended.

2.6 Batch Fermentation V/s Two stage feeding

The batch phase serves only to build up the biomass for producing Mevinolinic acid, a secondary metabolite that inhibits its own synthesis in the producing microfungus.

(i) A two-stage feeding strategy improves the rate of production of Mevinolinic acid by more than 50% when compared with conventional batch fermentation by *Aspergillus terreus.*

(ii) The feeding strategy consists of an initial batch/fed-batch phase and a semi-continuous culture dilution phase with retention of pelleted biomass in a slurry bubble column reactor.

(iii) Nitrogen free medium is to be used along with many minerals and biotin as an inducer that helped in increasing the production yield at 96 hrs.

The semi-continuous dilution phase provides nutrients to sustain the fungus, but prevents biomass growth by limiting the supply of essential nitrogen.

2.7 Genetic Regulation

lovE gene encodes a transcription factor regulatory protein with the typical binuclear Zn++ finger motif. Its disruption mutants did not produce Mevinolinic acid or intermediates, while the over expression resulted in increased metabolite production.

lovE regulates Mevinolinic acid production at the transcriptional level. So a direct effect on production can be expected. Targeting co-metabolites arising from a polyketide biosynthetic pathway, via site directed mutagenesis or gene shuffling, may direct the fungal machinery towards increased production of Mevinolinic acid than its co-metabolites.

Available options

(i) Over express lovE gene.

(ii) *Aspergillus terreus* has shown to produce several co-metabolites extracted from whole broth. The maximum production of Mevinolinic acid and sulochrin is seen on day seven and day five respectively. We may try to curb the production of co-metabolites viz. benzophenone, sulochrin and geodin by classical mutagenesis to favor Mevinolinic acid production.

3. MATERIALS AND METHODS

3.1 Microorganism: The fungal culture was maintained on slant or Roux bottle of PDA (Potato Dextrose Agar). The spores were obtained after incubating the slants/Roux bottle at 28 °C for 12 days. A suspension of spores was obtained by harvesting the slants/Roux bottle cultures with a sterile aqueous solution of 0.5% Tween 80. The spore count was determined by Automated Cell Counter (BIO -RAD TC-10).

3.2 Seed propagation: The vegetative propagation was carried out in 20L fermenter vessel containing 15L vegetative medium (Seed medium). It consists of (g/L): D-glucose, 11.50; Sucrose, 11.50; Skim milk, 7.00; Soya flour, 6.50; Yeast extract, 1.20; Citric acid, 2.50; Sodium acetate, 1.20; Potassium di-hydrogen phosphate, 0.050; Calcium carbonate, 1.50; PEG-400, 0.50; Glycerol, 5.00; UCN, 1.00 and make up volume with water to 1.00 liter. The medium was sterilized at 121±1°C for 30 minutes and was inoculated with the fungal spores from freshly harvested spore suspension. The vessel was run at 1.8 – 2.8 tip speed at 28°C for 36±4 hours. The fungal growth was used for inoculating the production fermenter vessel.

3.3 Production Phase:

Screening of production media: Five different culture media were studied for Mevinolinic acid production. The composition of each medium as given below:

M-1: (g/l): lactose, 10.00; yeast extract, 8.00; KH_2PO_4, 1.51; $MgSO_4 \cdot 7H_2O$, 0.52; NaCl, 0.40; $ZnSO_4 \cdot H_2O$, 0.001; $Fe(NO_3)_3 \cdot 9H_2O$, 0.002; biotin, 0.00004 and 1 ml trace element solution. The trace element solution contained per liter solution: $Na_2B_4O_7 \cdot 10H_2O$, 100 mg; $MnCl_2 \cdot 4H_2O$, 50 mg; 50 mg $Na_2MoO_4 \cdot 2H_2O$, and $CuSO_4 \cdot 5H_2O$, 250mg and make up with 1.00 liter of water [14].

M-2: (g/l): Glucose, 100.00; Corn steep liquor, 20.00 (v/v); Tomato paste, 5.00 (wet wt.), Beer yeast, 20.00 (wet wt.) and make up with 1.00 liter of water [15].

M-3: (g/l) : glucose, 40.00; milk powder ,15.00; soybean meal, 5.50; malt extract, 0.50; sodium acetate, 1.00; peptone, 1.00; NaCl, 0.20; $CaCO_3$,1.50; KH_2PO_4, 0.05; $MgSO_4.7H_2O$, 0.05 and make up with 1.00 liter of water [16].

M-4: (g/l): D-glucose, 78.00; Cotton seed meal, 7.00; Skim Milk, 55.00; Soya bean flour, 23.50; Citric acid, 10.50; Sodium acetate, 8.50; Magnesium sulfate, 0.50; Glycerol, 10.00; Calcium carbonate, 6.25; PEG-400, 3.00; Soya oil, 5.00; UCN, 1.00 and make up with 1.00 liter of water[17].

M-5: (g/l): Glucose, 50.00; yeast extract, 20.00; tomato paste, 30.00; oat meal, 20.00; sodium acetate, 10.00; ammonium sulfate, 5.00; potassium di-hydrogen phosphate, 2.00 and 10.00 ml trace element solution[18]. The composition of trace elements stock solution was (g\l); $FeSO_4.7H_20$, 1.00; $MnSO_4.4H_2O$, 1.00; $CuCl_2.2H_2O$, 0.025; $CaCl_2.2H_2O$, 0.10; H_3BO_3, 0.056; $(NH_4)_6MoO_4.H_2O$, 0.019; $ZnSO_4.7H_2O$, 0.20 and make up with 1.00 liter of water.

3.4 Extraction of Mevinolinic acid from mycelium: Harvested fermentation broth pH was adjusted to 3.5 - 4.0. Then cell mass was separated by centrifugation at 5000 rpm which was further extracted by toluene in 1:8 ratio. Rich solvent is then concentrated by vacuum distillation. Crude form further purified by Iso-propanol and then with Acetonitrile. Final product was obtained and dried

3.5 Mevinolinic acid estimation: Mevinolinic acid was estimated by whole broth extraction through HPLC (Water alliance 2695) using C8

column (YMC technology, 5µm, 250 x 4.6 mm). Acetonitrile: Water in the ratio of 65:35 (v/v) acidified with 0.1% ortho-phosphoric acid was used as mobile phase and was analyzed UV detector at 235 nm [19].

4.0 RESULTS AND DISCUSSION:

4.1 Screening of production medium: Five different fermentation media (M1-M5) were screened for the production of Mevinolinic acid by *A. terreus* in fermenter vessel (Fig. 1). It was found that fermentation media M-3 and M-4 were most suitable for the production of Mevinolinic acid by selected fungal strain with a slightly higher amount in the medium (M-4). So, the culture medium M-4 was selected for further studies and the maximum titer value was obtained 16.2 mg/gm. The maximum production of Mevinolinic acid in the medium (M-4) might be due to the presence of essential amino acids and mineral salts which were provided by the mixture of skim milk, cotton seed meal, soyabean flour and glycerol. This medium was found best due to presence of all the essential elements for fungal growth and Mevinolinic acid production. It is used similar culture medium for the production of Mevinolinic acid by *Aspergillus terreus* [14].

4.2 Optimization of seed transfer parameter: Different physical parameters were considered in this study. As per study, optimal parameters for transfer of seed medium to production medium were found out. Optimal age for seed transfer was 34 to 38 hours. pH was 5.75 ±0.25, PMV was 16 ±2. CO_2 concentration (volume %) was studied and showed 0.20 -0.22 at transfer of seed medium and O_2 concentration (volume %) was 15.0 -17.0 (Fig. 2).

4.3 Effect of seed transfer volume (%): The effect of Seed transfer volume percentage on the yield of Mevinolinic acid was studied by carrying out variations transfer volume of fermentation broth from 5% to 15% (Fig. 3). Highest production was observed when seed transfer volume was observed in between 9 -11 %. Seed transfer volume was considered 10% and the titer value was obtained 16.4 mg/gm as similar to 11%.

4.4 Effect of pH parameter: The effect of initial pH of production medium on the yield of Mevinolinic acid was studied by carrying out variations in the pH of fermenter broth from 5.0 to 8.0 by addition of D-glucose (Fig. 4). The highest yield was observed at higher pH values with optimal at pH 6.0 -7.0 and the titer value was obtained 16.2 mg/gm.

All the secondary metabolic activities normally occur at some specific pH and variation of pH during the fermentation process drastically affect the production[20]. It might be due to the fact that at pH 6.0, the permeability of cell membrane is enhanced by metallic ion for maximum production of Mevinolinic acid in the fermentation process [21].

4.5 Effect of Dissolve Oxygen (D.O.): The effect of D.O. percentage on the yield of Mevinolinic acid was studied by carrying out variations in D.O. of fermentation broth from 20% to 80%. Highest production was observed (Fig. 5) when D.O. was maintained 40% and the titer value was obtained 16.30 mg/gm. Indeed good results was found when D.O. was maintained in between 30 -40%[19].

5.0 CONCLUSIONS

In this study, *Aspergillus terreus* was used for the production of Mevinolinic acid; a cholesterol lowering drug. The strain showed 9.20 mg/gm of Mevinolinic acid production in the initial experiments. After optimization of the physical and nutritional conditions, *Aspergillus terreus* was capable of producing about 16.0 mg/gm Mevinolinic acid in the fermentation broth and mycelial extract, respectively.

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CONFLICT OF INTEREST: The author declares no conflict of interest.

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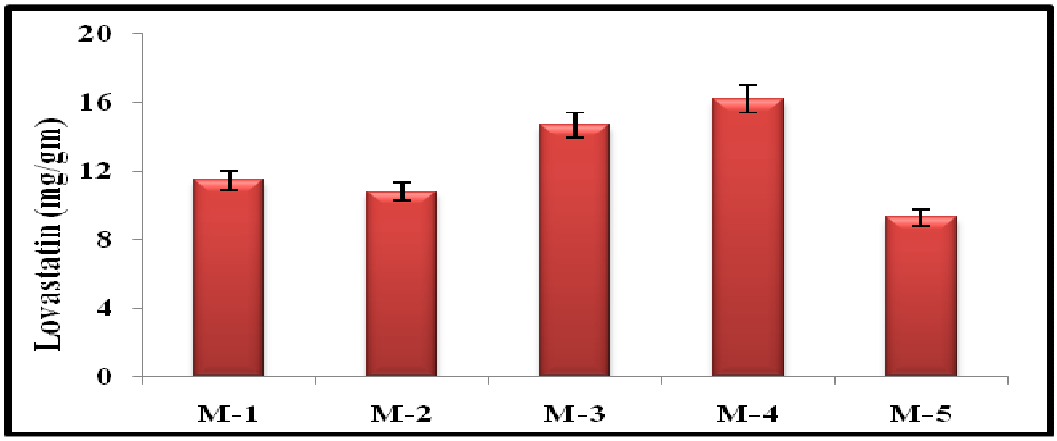

Fig 1: Screening of production medium for Mevinolinic acid fermenter batch by *A. terreus*.
"All values are the sum of three parallel replicates. Y-error bars indicate the standard error from the mean value".

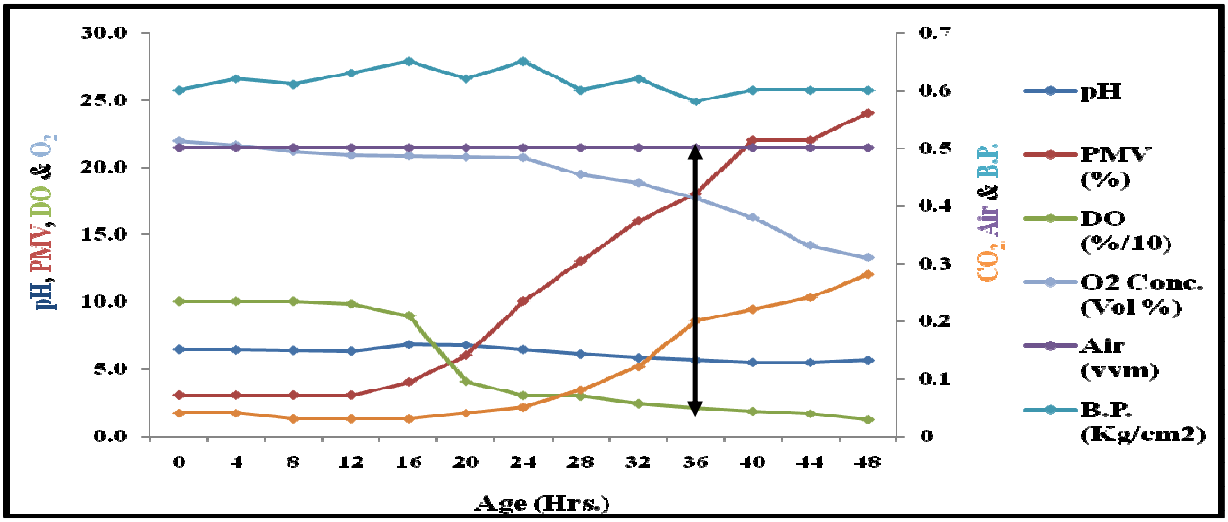

Fig 2: Standardization of seed transfer parameters

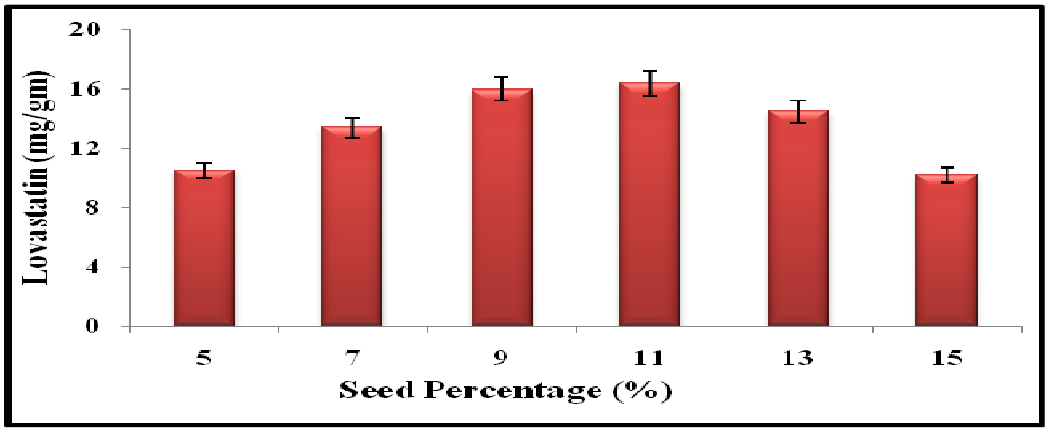

Fig 3: Screening of production medium for Mevinolinic acid fermenter batch by *A. terreus*.
"All values are the sum of three parallel replicates. Y-error bars indicate the standard error from the mean value".

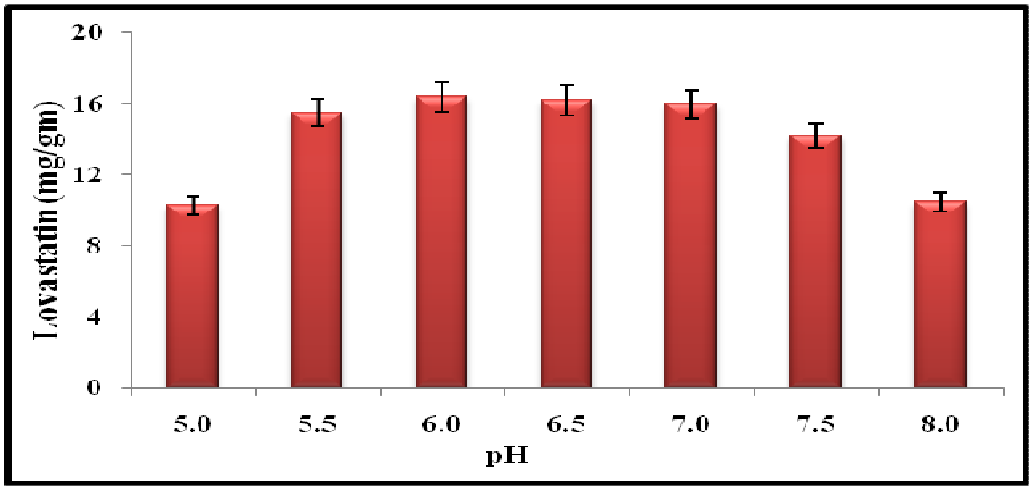

Fig. 4 Effect of initial pH of production medium on Mevinolinic acid by *A. terreus*.
"All values are the sum of three parallel replicates. Y-error bars indicate the standard error from the mean value"

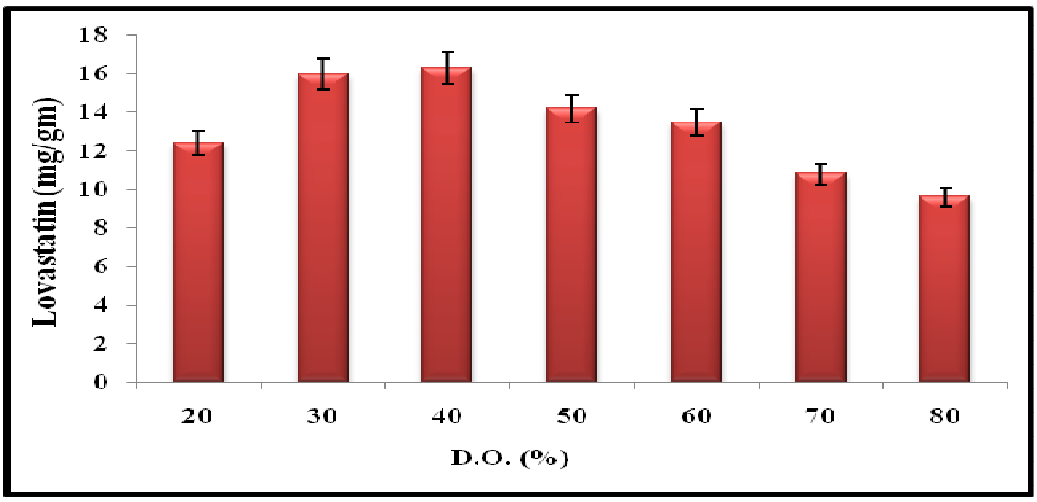

Fig. 5 Effect of initial Dissolve Oxygen (D.O.) (%) of production medium on Mevinolinic acid by *A. terreus*.
"All values are the sum of three parallel replicates. Y-error bars indicate the standard error from the mean value"