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PRODUCTION OF LOVASTATIN FROM *ASPERGILLUS TERREUS* THROUGH SUBMERGED FERMENTATION

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Abstract

The lab scale optimization of lovastatin production through submerged fermentation by *Aspergillus terreus* has been described. Fermentation experiments for the screening of fermentation media showed that *A. terreus* NRRL 265 utilized fermentation medium (M2) for maximum production of lovastatin i.e., 212.54 mg/L & 120.98 mg/L in the fermentation broth and mycelial extract, respectively. Similarly, when the effect of incubation period on statin production was studied, 144 h was found to be the best incubation period for statin production by this fungal strain. Further optimization of statin production was carried out by studying physical parameters such as the effect of incubation temperature, initial pH and nutritional parameters i.e., carbon sources, nitrogen sources, age and size of inoculum. An incubation temperature of 30ºC and the initial pH of 6.0 were found optimum for lovastatin production. As far as the carbon and nitrogen sources are concerned, 9% glucose, 2.5% corn steep liquor were found to be the best carbon and nitrogen sources, respectively in addition to 0.3% ammonium sulphate as inorganic nitrogen source. A 30 h old inoculum at a level of 5% was found best for lovastatin production by *A. terreus* NRRL 265. During the course of study, the maximum lovastatin production of 471.91 mg/L & 409.56 mg/L in the fermentation broth and mycelial extract respectively, was accomplished in shake flask fermentation by *A. terreus* NRRL 265.

Introduction

Statins are the secondary metabolic compounds produced by some fungal strains and are widely used to reduce elevated levels of cholesterol in blood plasma (Alberts, 1988). So, they are the most effective and suitable compounds in the treatment of hypercholesterolemia which is the one of the most important causes of deaths in the world (Goldstein & Brown, 1984). Statins act by competitively inhibiting the enzyme 3-hydroxy-3-methylglutaryle coenzyme A reductase (HMG-CoA) which is the necessary rate limiting enzyme for cholesterol synthesis (Alberts, 1990). The use of statins also prevents the development of atherosclerosis lesions and reduces cardiovascular morbidity, triglycerides levels and increases anti-atherogenic high-density lipoprotein in plasma (Vega & Grundy, 1998). The first statin identified from fungus *Penicillium citrinum* was the mevastatin (Endo *et al.*, 1977). Lovastatin was recommended by FDA as a cholesterol lowering drug in animals and human (Tobert, 1987). Lovastatin was isolated from *A. terreus* and the chemical changes of this fermentation derived drugs such as simvastatin and microbial modification led to drugs such as pravastatin. Lovastatin acts as a precursor for the production of another type of synthetic statin i.e., simvastatin.

Therapeutic applications and use of fungi have long been a tradition in the history of Asian countries (vamanu, 2013).Fungi are the most potent microorganisms for the statin production. Examples of some statin producing fungi include *Monascus rubber* (Endo, 1979), *M. purpureu* (Wang *et al.*, 2003), *M. paxi* (Manzoni & Rollini, 2002), *M. anka* (Su *et al.*, 2003), *Aspergillus terreus* (Hajjaj *et al.*, 2001), *A. flavipes* (Valera *et al.*, 2005), *A. flavus, A. fischeri, A. umbrosus, A. parasiticus, Acremonium chrysogenum, Byssoclamys fulva, Chaetomium viescens, Fusarium fujikuroi, Trichoderma longibrachiatum, T. viridae, Penicillium funiculosom* and *Rhizomucor miehei* (Samiee *et al.*, 2003).

During fermentation, various sources of carbon and nitrogen for the microorganisms are used. Lactose, glucose, glycerol as a source of carbon gives the maximum production of lovastatin (Szakacs *et al.*, 1998; Manzoni *et al.*, 1999). The fermentation medium with necessary and additional organic nitrogen sources such as beef extract, peptone, meat extract, yeast extract, corn steep liquor and inorganic nitrogen sources i.e., ammonium phosphate, ammonium chloride, ammonium sulphate and potassium nitrate, or ammonium nitrate was reported for the suitable production of statin from different fungal strains (Valera *et al.*, 2005). Among organic and inorganic nitrogen sources it has been considered that corn steep liquor and ammonium sulphate are suitable for the statins production. The different quantities of carbon and nitrogen sources are used in fermentation medium to have different effect on statin and microbial mass production (Lopez *et al.*, 2003). An appropriate initial pH is important for the progression and successful termination of fermentation (Ali *et al.*, 2005). The optimization of type and size of inoculum is very important in terms of substrate consumption by fungi and also provides the different ranges of statin production under control circumstances (Lopez *et al.*, 2003).

The main focus of the current study was to optimize different cultural and nutritional parameters for the production of lovastatin by *Aspergillus terreus* in shake flasks so that the optimized bioprocess can be transformed in to large scale production.

Materials and Methods

Microorganisms: The fungal strain of *Aspergillus terreus* NRRL 265 was kindly provided by Agricultural Research Service (ARS), USDA, USA which was used in the present studies for lovastatin in addition to some local isolates which were isolated from various soil samples from Lahore.

Shake flask fermentation

Inoculum preparation: The vegetative inoculum was prepared in 50 ml vegetative medium contained in 250 ml of Erlenmeyer flask and consisting of (g/L): tomato paste, 40; glucose, 12; corn steep liquor, 5 and 10 ml trace elements stock solution. The composition of trace elements stock solution was (g\L); $FeSO_4.7H_20$, 1; $MnSO_4.4H_2O$, 1; $CuCl_2.2H_2O$, 0.025; $CaCl_2.2H_2O$, 0.1; H_3BO_3, 0.056; $(NH_4)_6MoO_4.H_2O$, 0.019; $ZnSO_4.7H_2O$, 0.2 and water to 1 liter. The medium was autoclaved and was inoculated with the fungal spores from a slant. The flask was incubated at 30^0C for 24 h in a shaking incubator at 200 rpm. The fungal growth was used for inoculating the production flasks.

Fermentation batch: The fermentation experiments were carried out in 250 ml Erlenmeyer flasks containing 50 mL of fermentation medium consisting of (g/l); glucose, 50; yeast extract, 20; tomato paste, 30; oat meal, 20; sodium acetate, 10; ammonium sulfate, 5g; potassium dihydrogen phosphate, 2 and 10 ml trace element solution (Gunde-Cimerman *et al.,* 1973; Shinda, 1997). The composition of trace elements stock solution was (g/l); $FeSO_4.7H_20$, 1; $MnSO_4.4H_2O$, 1; $CuCl_2.2H_2O$, 0.025; $CaCl_2.2H_2O$, 0.1; H_3BO_3, 0.056; $(NH_4)_6MoO_4.H_2O$, 0.019; $ZnSO_4.7H_2O$, 0.2 and water to 1 liter. The pH was adjusted to 7.0 with 1N NaOH. The fermentation medium was autoclaved, cooled and inoculated with 2% of the vegetative inoculum as prepared above. The flasks were incubated in a rotary shaker incubator at 200 rpm at 28°C for 7days. After completion of the fermentation, the fermentation broth and fungal mycelium were used to test the production of statins.

Extraction of statin from mycelium: The fungal mycelium was oven dried at 40^{o}C for 24 hrs and was used for the extraction of statin in methanol: water mixture (1:1, v/v) in 100 ml Erlenmeyer flask keeping the flask at 200 rpm for 2 hrs. This mixture was centrifuged at 10,000 rpm for 10 min and supernatant was obtained which was further filtered through 0.45µm nitro cellulose membrane filter for the estimation of statins by HPLC.

Statin estimation: Lovastatin was estimated in the fermentation broth and the mycelial extracts through HPLC (Perkin Elemer 200 series, USA) using C-18 column (SpherSIL, 0.5µm, 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. The HPLC system was equipped with peristaltic pump and a UV detector. The mobile phase flow rate was maintained at 1.5 ml/min and statin was detected at 235 nm (Samiee *et al.,* 2003).

Screening of culture media: Five different culture media were screened for statin production. The composition of each medium is given as:

M 1: (g/l): lactose, 10; yeast extract, 8; 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 (Lopez *et al.,* 2003).

M 2: (g/l): Glucose, 100; Corn steep liquor, 20 (v/v); Tomato paste, 5 (wet wt.), Beer yeast, 20 (wet wt.) (Novak *et al.,* 1997).

M 3: (g/l) : glucose, 40; milk powder ,15; soybean meal, 5.5; malt extract, 0.5; sodium acetate, 1.0; peptone, 1.0; NaCl, 0.2; $CaCO_3$,1.5; KH_2PO_4, 0.05; $MgSO_4.7H_2O$, 0.05 (Gupta *et al.,* 2007).

M 4: (g/l): glucose, 45; mono-hydrate sodium glutamate, 12.5; KH_2PO_4, 5; K_2HPO_4, 5; $FeSO_4{\cdot}\ 7H_2O$, 0.2; $MnSO_4\ {\cdot}\ 4H_2O$, 0.1; $ZnSO_4$, $7H_2O$, 0.2; $MgSO_4\ {\cdot}\ 7H_2O$, 0.1; $CaCl_2\ {\cdot}\ 2H_2O$, 0.02; $CuCl_2\ {\cdot}\ 2H_2O$, 0.005; H_3BO_3, 0.011 and $(NH_4)_6Mo_7O_{24}\ {\cdot}\ 4H_2O$, 0.005 **(**Hajjaj *et al.,* 2001).

M 5: (g/l): glucose, 50; yeast extract, 20; tomato paste, 30; oat meal, 20; sodium acetate, 10; ammonium sulfate, 5g; potassium dihydrogen phosphate, 2 and 10ml trace element solution (Shinda, 1997). The composition of trace elements stock solution was (g\l); $FeSO_4.7H_20$, 1; $MnSO_4.4H_2O$, 1; $CuCl_2.2H_2O$, 0.025; $CaCl_2.2H_2O$, 0.1; H_3BO_3, 0.056; $(NH_4)6MoO_4.H_2O$, 0.019; $ZnSO_4.7H_2O$, 0.2 and water to 1 liter.

Results and Discussion

Screening of fungal isolates: *Aspergillus terreus* NRRL 265 alongwith 12 local soil isolates were tested for statin production in shake flasks. Maximum production of lovastatin was shown by *Aspergillus terreus* NRRL 265 i.e., 120mg/L & 96.00mg/L in its culture broths and mycelial extracts, respectively. *Aspergillus terreus* has also been used for the production of similar metabolites (Nahar *et. al.,* 2008).

Screening of culture media: Five different fermentation media (M1-M5) were screened for the production of lovastatin by *A. terreus* NRRL 265 in shake flasks (Fig. 1). It was found that fermentation media M2 and M4 were most suitable for the production of lovastatin by selected fungal strain with a slightly higher amount in the medium (M2). So, the culture medium M2 was selected for further studies. The maximum production of lovastatin in the medium (M2) might be due to the presence of essential vitamins *i.e.,* biotin, inositol and thiamine (B1), amino acids, arginine, lysine and tyrosine and mineral salts of K, P, Mg, Zn and Mo which were provided by the mixture of tomato paste, corn steep liquor and beer yeast. This medium was found best due to presence of all the essential elements for fungal growth and lovastatin production. Lopez *et al.,* (2003) used somewhat similar culture medium for the production of lovastatin by *Aspergillus terreus.*

Effect of incubation period: The effect of incubation period on the lovastatin production by *A. terreus* was studied by varying the incubation period of fermentation flasks from 24 to 192 hrs (1-8 days). The production of lovastatin after 24 hrs of incubation was 89.23 mg/l and 63.56 mg/l in the fermentation broth and mycelial extract, respectively and increased with increasing incubation period reaching to the maximum value of 220.78 mg/l and 189.96 mg/l after 144 hrs of incubation in the fermentation broth and mycelial extract, respectively (Fig. 2). It declined when further incubation was given to the fungal strain in the shake flasks and after an incubation period of 192 hrs, the yield of lovastatin was minimum showing an amount of 110.36 mg/l and 78.02 mg/l in the fermentation broth and the mycelial extract, respectively. The growth of the fungal strain as indicated by dry mycelial mass was also maximum after 144 hrs and started to decrease as incubation period was increased. Therefore, an incubation period of 144 hrs was found to be the best for lovastatin production by *A. terreus* NRRL 265. Lopez *et al.,* (2003) found 150 hrs as the optimum incubation period for lovastatin production by *Aspergillus terreus*.

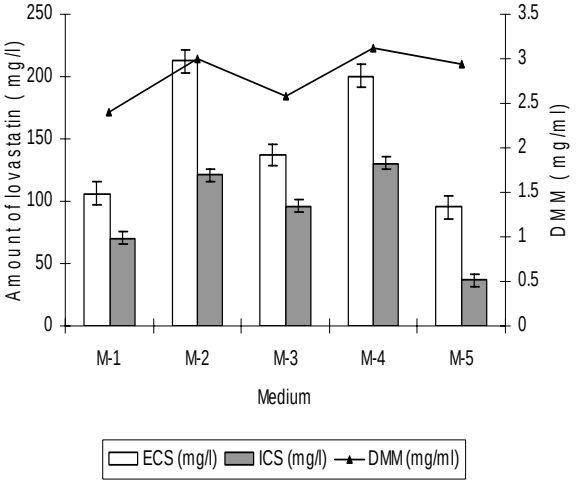

Fig. 1. Screening of culture media for lovastatin production by *A. terreus* in shake flask fermentation.
"All values are the sum of three parallel replicates. Y-error bars indicate the standard error from the mean value". **Abbreviations:** ECS: Extra cellular statin; ICS: Intra cellular statin; DMM: Dry mycelial mass.

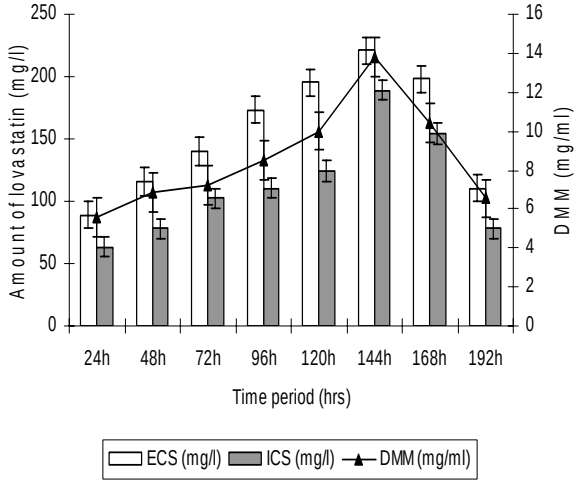

Fig. 2. Effect of incubation period on lovastatin production by *A. terreus* NRRL 265 in shake flasks fermentation.
"All values are the sum of three parallel replicates. Y-error bars indicate the standard error from the mean value". **Abbreviations:** ECS: Extra cellular statin; ICS: Intra cellular statin; DMM: Dry mycelial mass.

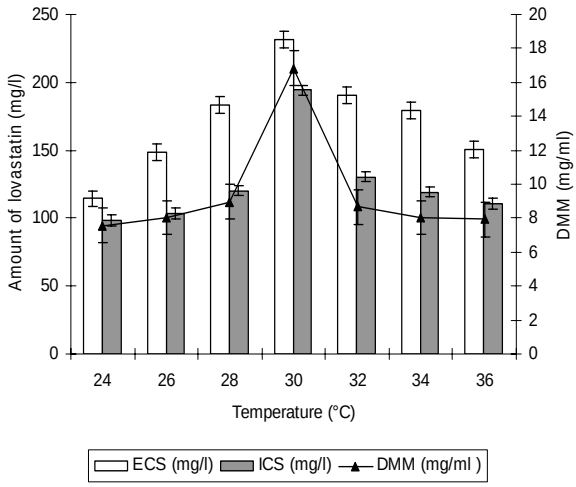

Fig. 3. Effect of incubation temperature on lovastatin production by *A. terreus* NRRL 265 in shake flasks fermentation.
"All values are the sum of three parallel replicates. Y-error bars indicate the standard error from the mean value".

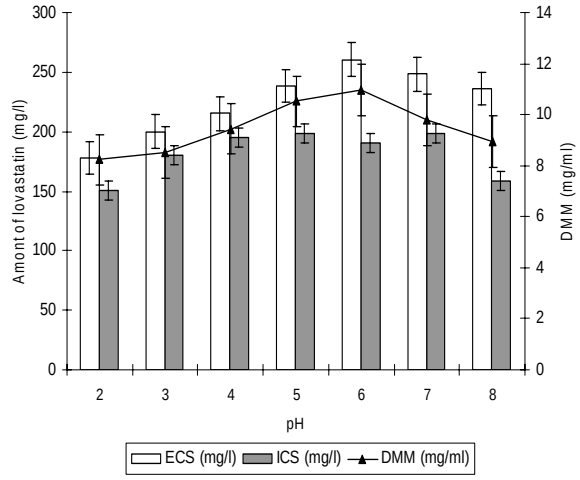

Fig. 4. Effect of initial pH of culture medium on lovastatin production by *A. terreus* in shake flasks fermentation.
"All values are the sum of three parallel replicates. Y-error bars indicate the standard error from the mean value".

Effect of incubation temperature: The effect of different incubation temperatures on the lovastatin production by *A. terreus* NRRL 265 was studied by culturing the shake flasks in the temperatures ranging from 24 to 36°C (Fig. 3). From the results, it was observed that there was a gradual increase in lovastatin production when the incubation temperature was increased from 24°C to 30°C. The maximum lovastatin production, 231.50 mg/l & 194.00 mg/l was observed at 30°C in the fermentation broth and mycelial extract, respectively. The lovastatin production was gradually decreased with an increase of temperature from 30°C to 36°C. The minimum lovastatin production was observed at 36°C i.e., 150.47 mg/l and 110.50 mg/l in the fermentation broth and mycelial extract, respectively. So, the optimum incubation temperature for lovastatin production was found to be 30°C. The maximum growth of fungal strain as indicated by dry cell mass of 17.2 mg/ml was also observed at 30°C. The maximum production of lovastatin at 30°C might be due to the fact that this temperature is best for the sporulation, growth and proliferation of mycelial mass for the production of secondary metabolites. In this study, the optimal incubation temperature (30°C) found for lovastatin production is closely related to the work of Samiee *et al.*, (2003), Gupta *et al.*, (2007) and Atalla *et al.*, (2008) who have also reported the lovastatin production at 28-30°C.

Effect of initial pH: The effect of initial pH of culture medium on the production of lovastatin was studied by varying the initial pH of culture media from 2.0 to 8.0 (Fig. 4). Lowest lovastatin production was observed at pH 2.0 i.e., 178.18 mg/l & 150.21 mg/l in the fermentation broth and mycelial extract, respectively. The production was started to increase at higher pH values and maximum lovastatin production i.e., 260.38 mg/l & 190.69 mg/l in the fermentation broth and mycelial extract, respectively was observed at pH 6.0 and then it was decreased by increasing the initial pH above 6.0. Maximum fungal growth was also observed as dry cell mass of 10.96 mg/ml at pH 6.0. Therefore, the best result for lovastatin production was achieved at pH 6.0 in shake flask culture and it was maintained during further studies. All the secondary metabolic activities normally occur at some specific pH and variation of pH during the fermentation process drastically affect them (Kysilka, 1993). 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 lovastatin in the fermentation process (Madan & Thind, 2000).

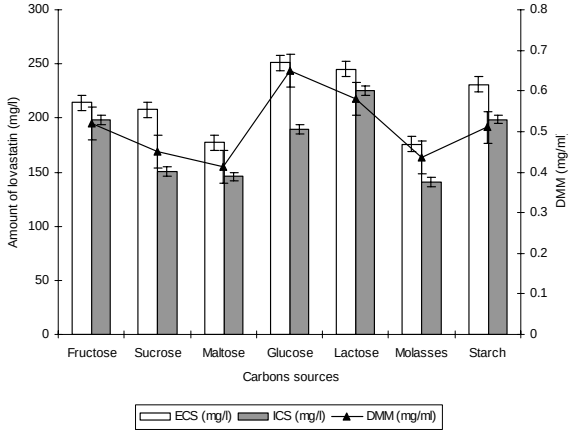

Fig. 5a. Evaluation of different carbon sources for lovastatin production by *A. terreus* in shake flasks fermentation.
"All values are the sum of three parallel replicates. Y-error bars indicate the standard error from the mean value".

Screening of different carbon sources: Different carbon sources including glucose, maltose, molasses, lactose, sucrose, fructose and starch were evaluated for the maximum lovastatin production by *Aspergillus terreus*. The results showed that glucose gave maximum lovastatin production i.e., 250.04 mg/l & 189.36 mg/l in fermentation broth and mycelial extract, respectively (Fig. 5a) followed by lactose with a slight difference. Another experiment was carried out to optimize the concentration of glucose (4-10%) in the culture medium. Yield of lovastatin was increased as the glucose concentration was increased and reached maximum (290.21 mg/l & 253.33 mg/l) at 9% glucose concentration as shown in the figure (Fig. 5b). Therefore, 9% glucose was selected as the most suitable concentration for the production of lovastatin by *A. terreus* due to the fact that it is easily available carbon source and it oxidized very rapidly in the cells thus act as a readily available source of energy. Hajjaj *et al.*, (2001) have also reported glucose as a suitable carbon source for lovastatin production.

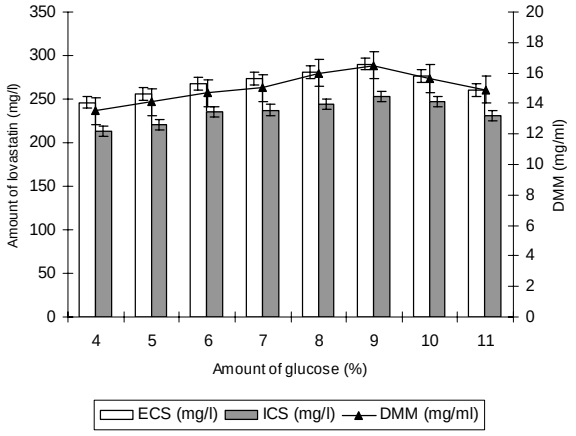

Fig. 5b. Effect of different concentrations of glucose on lovastatin production by A. *terreus* in shake flasks fermentation.
"All values are the sum of three parallel replicates. Y-error bars indicate the standard error from the mean value".

Evaluation of different nitrogen sources: Different nitrogen sources i.e. organic and inorganic such as meat extract, corn steep liquor, urea, peptone, beef extract and yeast extract were evaluated for the maximum lovastatin production by *A. terreus* NRRL 265. Of all the organic sources, corn steep liquor gave maximum lovastatin production i.e., 311.02 mg/l & 280.01 mg/l in both the fermentation broth and mycelial extract, respectively (Fig. 6a). The growth of *A. terreus* NRRL 265 as indicated by dry mycelial mass was also low in meat extract but was maximum (20.41 mg/ml) in case of corn steep liquor. The amount of corn steep liquor (0.5-3.5%) was further optimized for the production of lovastatin. The results showed that corn steep liquor at a concentration of 2.5% gave maximum production of lovastatin i.e., 351.23 mg/l & 298.37 mg/l in the fermentation broth and mycelial extract, respectively as shown in the results (Fig. 6b). Among different inorganic sources tested, ammonium sulphate showed the best lovastatin yield i.e., 392.2 mg/l & 373.66 mg/l in the fermentation broth and mycelial extract, respectively (Fig. 6c). Ammonium sulphate concentration (0.1% to 0.6%) was further optimized for the enhanced production of lovastatin. It was noted that ammonium sulphate at 0.3% concentration gave maximum yield of lovastatin i.e., 411.0 mg/l & 349.23 mg/l in fermentation broth and mycelial extract, respectively (Fig. 6d).

Effect of age and size of inoculums: The effect of inoculum age and size on the production of lovastatin by *A. terreus* NRRL 265 was studied. Different ages of the fungal vegetative inoculum ranging from 12-46 hrs were tested for lovastatin production (Fig. 7a). Maximum lovastatin production i.e., 442.35 mg/l & 396.12 mg/l in fermentation broth and mycelial extract, respectively was obtained using inoculum age of 30 hrs. However, maximum dry mycelial mass 22.7 mg/ ml was found at 30 hrs old inoculum was used.

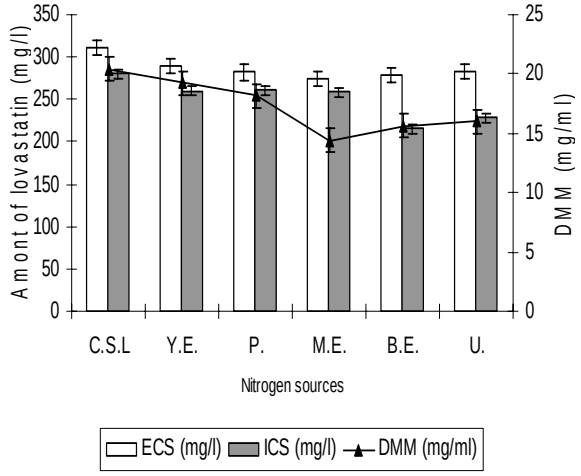

Fig. 6a. Effect of different organic nitrogen sources on lovastatin production by A. *terreus* NRRL 265 in shake flasks fermentation. "All values are the sum of three parallel replicates. Y-error bars indicate the standard error from mean". **Abbreviations:** ECS: Extra cellular statin; ICS: Intra cellular statin; DMM: Dry mycelial mass. CSL: Corn steep liquor; YE: Yeast extract; P: Peptone; ME: Meat extract; BE: Beaf extract; U: Urea.

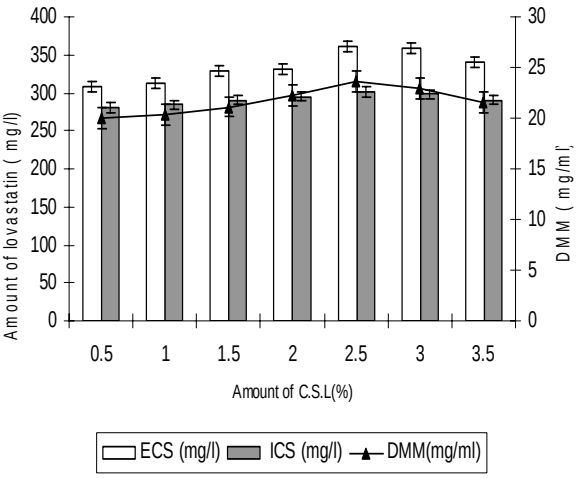

Fig. 6b. Effect of different concentrations of corn steep liquor on lovastatin production by *A. terreus* in shake flasks fermentation "All values are the sum of three parallel replicates. Y-error bars indicate the standard error from mean"

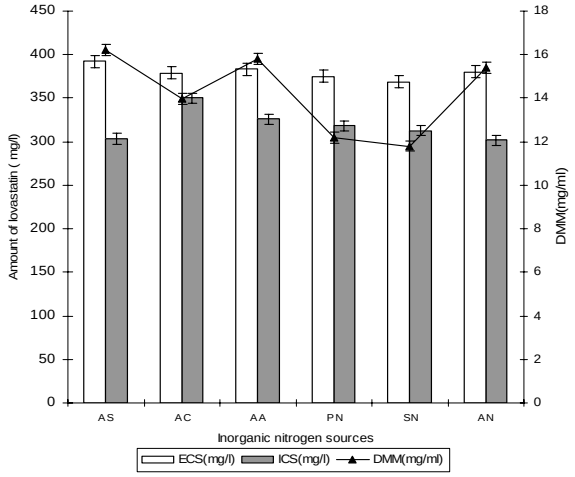

Fig. 6c. Effect of different inorganic nitrogen sources on lovastatin production by *A. terreus* in shake flasks fermentation. "All values are the sum of three parallel replicates. Y-error bars indicate the standard error from the mean values". **Abbreviations:** ECS: Extra cellular statin; ICS: Intra cellular statin; DMM: Dry mycelial mass; AS: Ammonium Sulphate; AC: Ammonium Chloride; AA: Ammonium Acetate; PN: Potasium Nitrate; SN: Sodium Nitrate; AN: Ammonium Nitrate.

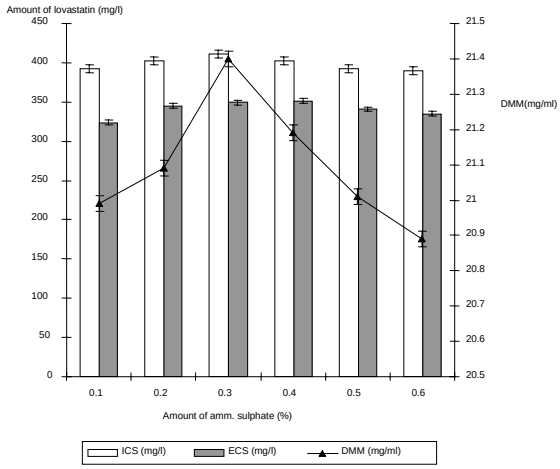

Fig. 6d. Effect of different concentrations of ammonium sulphate on lovastatin production by *A. terreus* in shake flasks fermentation. "All values are the sum of three parallel replicates. Y-error bars indicate the standard error from mean".

Another experiment was carried out to optimize the size of inoculum ranging from 1–7% for the lovastatin production (Fig. 7b). The results showed that maximum lovastatin production was 471.91 mg/l & 409.56 mg/l in the fermentation broth and mycelial extract, respectively when 5% inoculum was used to inoculate the fermentation flasks. Maximum fungal growth was also observed as dry cell mass of 23.12 mg/ml at 5% inoculum size. Therefore, a 30 hrs old vegetative inoculum at level of 5% (v/v) was selected for the maximum production of lovastatin by *A. terreus* NRRL 265. The findings about the size of inoculum (5% v/v) are different from the other workers as Hajjaj *et al.*, (2001) and Samiee *et al.*, (2003) reported 3% and 10% inoculum, respectively as best for lovastatin production by *A. terreus*.

Conclusions

In the present study, *Aspergillus terreus* NRRL 265 was used for the production of lovastatin; a cholesterol lowering drug. The strain showed 120.40 mg/L of lovastatin production in the initial experiments. After optimization of the physical and nutritional conditions, *Aspergillus terreus* was capable of producing about 4 fold increased lovastatin i.e., 471.91mg/L & 409.56 mg/L in the fermentation broth and mycelial extract, respectively.

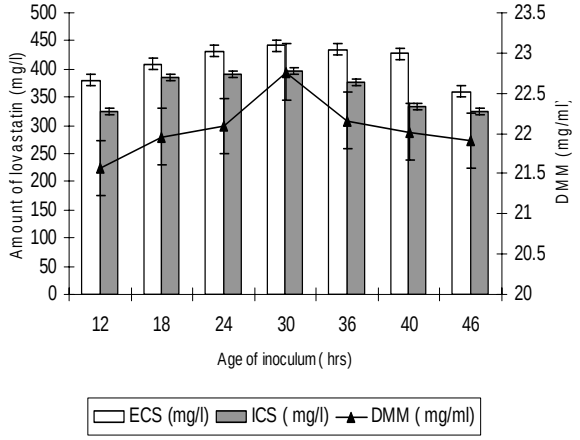

Fig. 7a. Effect of different ages of inoculum on the lovastatin production by *A. terreus* NRRL 265 in shake flasks fermentation. "All values are the sum of three parallel replicates. Y-error bars indicate the standard error from mean".

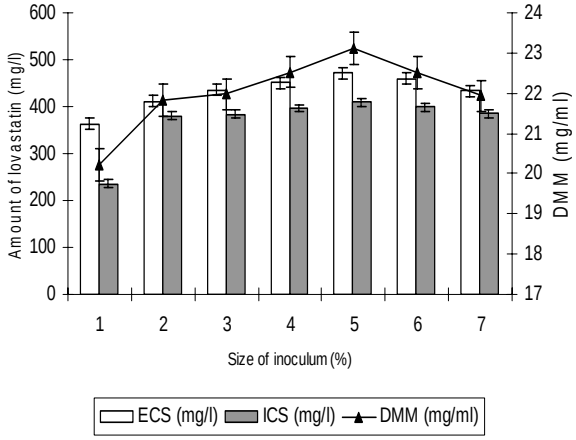

Fig. 7b. Effect of different sizes of inoculum on lovastatin production by *A. terreus* NRRL 265 in shake flasks fermentation. "All the values are the sum of three parallel replicates. Y-error bars indicate the standard error from mean".

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