Studies on Process Parameters for Production of Lovastatin

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Research Article

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ABSTRACT :

Lovastatin is a natural secondary metabolite produced by *Aspergillus terreus*. Optimization of different process parameters like pH of medium, incubation period and selection of different carbon sources etc. was investigated for maximum production of lovastatin using *Aspergillus terreus* NCIM 657. The kinetics of lovastatin production was also investigated up to fourteen days of production under batch mode. It was found that a temperature of 28 ^{0}C for incubation, a medium pH 6.5 and an agitation speed of 180rpm for seven days yielded maximum concentration of lovastatin. It was also observed that glucose as a sole carbon source resulted in diminished production of lovastatin as compared to cases where other carbon sources were supplemented in medium along with glucose. Thin layer chromatography, Fourier Transform Infrared spectroscopy analysis confirmed the presence of lovastatin in the sample produced by *A. terreus* NCIM 657.

Keywords: *Aspergillus*, Cardiovascular diseases, Cholesterol, HMG CoA reductase, Lovastatin, Thin Layer Chromatography.

INTRODUCTION:

According to the World Health Organization, Cardiovascular diseases are the leading cause of death and an estimate showed that about 17.45 million people died from these diseases accounting for approximately 30% of global mortality in 2005. This was due to high level of cholesterol in plasma, as hypercholesterolemia is a primary risk factor for heart disease called as atherosclerosis (Kannel*et al.* 1961). Cholesterol is a sterol which is an essential structural component required to establish proper membrane permeability and fluidity. The main role of cholesterol is to maintain normal body functions. It has been observed that one third of the total body cholesterol is derived from diet while the rest two thirds are synthesized by the liver and to a lesser extent by other organs (Furberg 1999; Alberts *et al.* 1980). Hence, inhibiting the synthesis of cholesterol is an important strategy to lower cholesterol levels in blood. Lovastatin ($C_{24}H_{36}O_5$) also known as Mevinolin, Monacolin K, and Mevacor, is a Class II Pro-drug which lowers blood cholesterol. It competitively inhibits the HMG CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA reductase) enzyme, the rate limiting enzyme of cholesterol biosynthesis pathway. HMG CoA reductase is involved in conversion of HMG CoA to mevalonate in cholesterol biosynthetic pathway. The enzyme associates with lovastatin as substrate with higher concentration than HMG CoA and blocks the production of mevalonate, lowering the concentration of cholesterol particularly LDL (low density lipoproteins or bad cholesterol) while slightly increasing the HDL (high density lipoproteins or good cholesterol) level thus preventing the plaque formation inside the blood vessels. Lovastatin can also be used for in vivo tumor suppression due to synthesis of non-sterol isoprenoid compounds. This natural compound is produced as a secondary metabolite by many filamentous fungi including *Penicillum*sp., *Monascusruber,* and *Aspergillus terreus.* But for commercial production, *Aspergillus terreus* is the most widely used mold and batch fermentation is preferred mode of production process (Bizukoj*et al.* 2009, Casas Lo´ pez JL*et al.* 2004). The present work highlighted the optimization of various process parameters for maximum production of lovastatin using a wild strain *Aspergillus terreus* NCIM 657 and the recovery of the product.

MATERIALS AND METHODS

CULTURE CONDITION

Maintenance Medium:

The fungus *A. terreus* NCIM 657 was procured from National Collection of Industrial Microbiology, Pune, India and was periodically grown in Potato Dextrose Agar (PDA) plate at 28° C for 5 days. The slants were kept at 4° C for further use. For all the experimental work, the fresh culture was used to inoculate the production media. All other chemicals of laboratory and analytical grades were used during the optimization process.

Production Medium:

The production of lovastatin was carried out as batch mode in shake flask under agitation speed of 170-180 rpm for 7-8

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days at 28° C. For this, 250 ml Erlenmeyer flask containing 50 ml of media was prepared. The pH of production medium was adjusted to 6.5 before sterilization. 6.0 mm block of freshly grown mold from PDA plate was aseptically transferred to the production media and production of lovastatin was monitored. At the end of production, pH of broth, cell biomass, crude lovastatin concentration as well as zone of inhibition by product against indicators (*Candida albicans* and *Neurosporacrassa*) were recorded. The maximum absorbance of lovastatin (λ_{max}) was also investigated. The production medium contained (per liter): Glucose - 45 g; Monohydrate sodium glutamate - 12.6 g; KH_2PO_4 - 4 g; K_2HPO_4 - 5 g; $FeSO_4.7H_2O$ - 0.2 g; $MnSO_4.4H_2O$ - 0.1 g; $MgSO_4.7H_2O$ - 0.1 g; $ZnSO_4.7H_2O$ - 0.2 g; $CaCl_2.2H_2O$ - 20 mg; $CuCl_2.2H_2O$ - 5 mg; H_3BO_3 - 11mg and $(NH4)_2MoO_4$ - 5mg (Gupta *et al.* 2009).

Optimization of cultural conditions

Effects of different pH on production of Lovastatin:

The effect of different pH values on the production of lovastatin was studied by adjusting the pH of fermentation medium in the range from 5.5 to 7.0 using 1N hydrochloric acid and 1N sodium hydroxide and inoculated with the freshly grown mold after sterilization of the medium. The process of production was observed for 7 days at 28° C under agitation speed of 180rpm and finally the analysis of production process was carried out as described previously. Kavitha*et al.*2012 has also studied the effects of pH of culture medium on Lovastatin production. To evaluate the effect, she has adjusted the pH in the range of 4.0 to 9.0 with 1N HCL or 1N NaOH, while keeping other parameters at their optimum.

Effects of different carbon sources on production of Lovastatin:

Six different media containing different carbon sources were tested for their abilities to influence growth and lovastatin production by the *A. terreus* NCIM 657 (Table -1). The different flasks containing 50 ml of these media were autoclaved after adjusting pH to 6.5 and inoculated for 7 days at 28° C under an agitation speed of 180rpm. At the end the fermentation, the production of lovastatin and other assays were determined. Zhihuajia *et al.*(2009) has also studied the effects of carbon sources on fungal morphology and lovastatin biosynthesis. In his studies, he has used five different kinds of carbon sources including Glucose, Glycerol, Sucrose, Lactose and soluble starch. The pH of the medium was kept at 6.5 in his studies.

Table 1: Media for lovastatin production with different carbon sources

Serial No.	Carbon sources (g/L)
Media I	Glucose - 45
Media II	Glycerol - 45
Media III	Lactose - 45
Media IV	Starch - 45
Media V	Glucose - 25, Lactose - 10, Starch - 10L
Media VI	Czapek Dox Broth

Effects of incubation periods on production of Lovastatin:

For the kinetic study of production of lovastatin by *A. terreus*, 50 ml of optimized medium in different flasks of 500 ml capacity was sterilized and inoculated at standard conditions. The fermentation broth was harvested at different time intervals (0, 1, 3, 5, 7, 10, 14, 18 days respectively) for analysis of lovastatin production. In each case, dry cell weight, crude concentration of produced lovastatin and carbon estimation was measured .Arjumand Ahmed *et al.* 2013has investigated the effect of incubation period for statin production by *A. terreus*. Spectrum analysis, thin layer chromatogram and bioassay against *Candida albicans* and *Neurospora crassa* were also performed.

EXTRACTION OF LOVASTATIN FROM CULTURE BROTH:

At the end of fermentation process of the lovastatin, the broth was acidified to pH 3.0 with concentrated H_2SO_4/ HCl for lactonization purpose followed by extraction with equal volume of ethyl acetate under shaking condition for 2h at 28° C. The broth was subsequently centrifuged at 10,000 rpm for 15 min for collection of organic phase. The organic phase was separated, dried and dissolved in 2 ml of acetonitrile for estimation of lovastatin. In other experiment, different solvents such as diethyl ether, ethyl acetate, ethyl acetate: cyclohexane (65:35) and butyl acetate respectively were used to find out the most suitable solvent for maximum recovery of lovastatin. The concentration of lovastatin in broth and in the crude samples was determined against the standard lovastatin procured from Hi-media (India). Nigam *et al.* (2015) has mentioned the extraction process of lovastatin by acidifying the broth to pH 3.0, which was later extracted with two volume of Ethyle Acetate after keeping it in rotary shaker at 170 rpm for 2 h at 28° C.

BIOMASS MEASUREMENT:

The fungal biomass was separated after submerged fermentation by filtration using pre-weighted filter paper. The filter paper containing fungal biomass was dried till constant weight at 50° C and finally the weight of fungal biomass was calculated as dry cell biomass.

SAMPLE ANALYSIS

Estimation of lovastatin:

Different aliquots (concentrations) of standard lovastatin were prepared in acetonitrile for measurement of zone of inhibition against indicators organisms *Candida albicans* and *Neurospora crassa* grown on their respective media at 28° C for 24-48h and a standard plot was constructed. The zone of inhibition produced from the organism was calculated from the standard plot. 50 microliter of standard as well crude samples was applied in a well of 6 mm diameter on agar plate for the present study. Similarly, the λ_{max} of lovastatin and thin layer chromatography (TLC) were also performed. For TLC analysis a silica gel-G 254 coated TLC plates was used. The plate was initially activated by heating in micro-oven for 1-2 min. Different extracted samples of lovastatin from production medium along with known concentration of standard lovastatin was spotted above 15 mm of the solvent system in chromatographic chamber. The dichloromethane: ethyl acetate (70:30 v/v) was used as solvent system. The visualization of the developed spots was observed under UV chamber and R_f values were recorded and compared with the standard. Fourier transform infrared spectroscopy (FTIR) analysis was also attempted

for the confirmation of extracted product. Upendra *et al.* (2013) has also studied the production, extraction and characterization of lovastatin by HPLC and FTIR studies.

RESULTS AND DISCUSSION

The preliminary production of lovastatin by *Aspergillus terreus* NCIM 657 was studied as batch mode under agitation using glucose as a sole carbon source at pH of 6.5 and temperature of 28°C, respectively. The spectrum analysis (λ_{max}) of crude lovastatin is presented in Figure 1 along with standard lovastatin. Pure lovastatin has three different absorption maxima at 238nm in UV-visible spectrophotometry, which suggests better identification of lovastatin from other compounds. The characteristics of the peaks could be due to the presence of diene. The absorption spectra of both lactone form and hydroxy acid form of lovastatin in a mixture appear similar.

The quantification of lovastatin by this protocol resulted to a concentration of 0.159 g/L of statin. The presence of other peaks in the sample during spectrometric analysis is due to the existence of impurities or other unidentified compounds that have been extracted with the solvent. The results obtained confirmed the presence of lovastatin at 238nm as analyzed by Upendra *et al.* (2013).

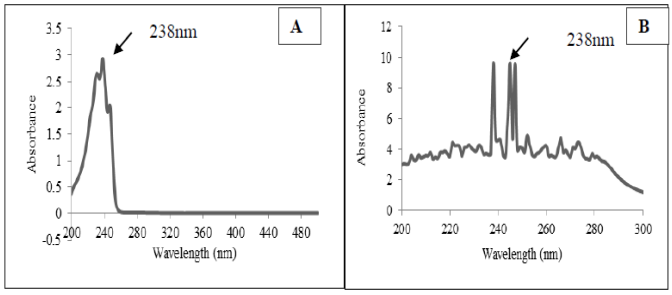

Figure 1: UV Spectrometric analysis of standard lovastatin (A) and crude lovastatin produced by *A. terreus* NCIM 657(B).

The identification of lovastatin was also carried out by thin layer chromatography and a R_f value of 0.58 was observed corresponding to the standard lovastatin.

Effect of different pH on the production of lovastatin:

The effect of different pH of the production medium for maximum production of lovastatin was given in Figure 2. The figure revealed that maximum production of lovastatin was observed at pH of 6.5 (optimal) and then it decreased. It was further noticed that production medium with pH 7.0 also favored synthesis of lovastatin. But, Kavitha *et al.* (2012) reported the optimum pH of 5.0 for lovastatin production using solid state fermentation process. At this pH, the maximum amount of Lovastatin was 0.87 mg/g. Szakaes*et al.* (1998) reported maximum production of lovastatin in sweet sorghum pulp at pH 6.2 while Valera *et al.* (2005) observed maximum lovastatin production at pH 5.0 using *A. flavipes* as producing strain using wheat bran as substrate.

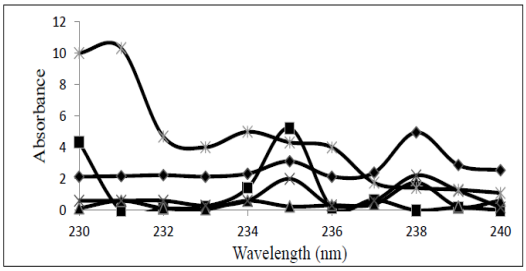

Figure 2: Effect of different pH on production of lovastatin, pH 5.5 (■); 6.0 (▲); 6.5 (X); 7.0 (*) and standard (♦)

Thin layer chromatography analysis:

A quantity of 20 µl of the crude extract from production medium with different pH was applied on silica gel-G 254 coated TLC plates along with known concentration of standard lovastatin. After about 30 min, the plate was removed from the chamber and dried at room temperature. The developed spot was marked under UV chamber and R_f values were calculated and shown in Table 2. The TLC profiling at different pH of medium also confirmed the presence of lovastatin at the optimal pH of 6.5 (Figure 3).

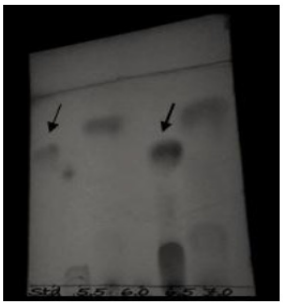

Figure 3: TLC of lovastatin at different pH

Table 2: Rf values of lovastatin at different medium pH

Sample	Rf
pH 5.5	0.53
pH 6.0	0.63
pH 6.5	0.58
pH 7.0	0.64
Standard	0.58

Kinetics of production of lovastatin by *A. terreus* NCIM 657:

For the kinetics study of production for lovastatin, 50 ml of sterile medium in different Erlenmeyer flasks was inoculated aseptically and fermentation process was monitored at different incubation periods (0-18 days) at optimal conditions. At the end of the incubation periods, ach flask was analyzed for lovastatin production (concentration), dry cell biomass, glucose consumption respectively. The result of the production profile is presented in Figure 4. The effect of incubation period on the lovastatin production by *A. terreus* was studied by varying the incubation period of fermentation flasks from 0 to 18 days. The production of lovastatin (crude concentration on weight basis) was increased till 7 days of fermentation. After that it was gradually decreased. The growth of fungal cell as indicated by the dry cell weight was also maximum at day 7. Therefore, an incubation period of 7 days was found to be the optimum for lovastatin production by *A. terreus*.

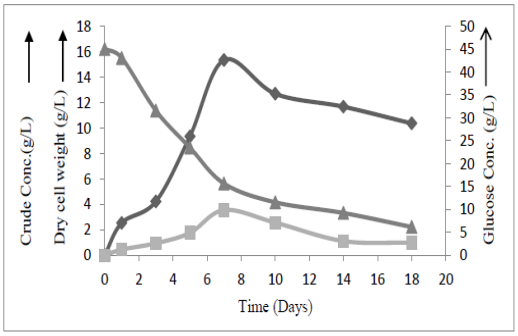

Figure 4: Kinetics of lovastatin production process. Dry cell weight (♦), Crude conc. (■), Glucose conc. (▲) with fermentation time

The effect of incubation period on the lovastatin production by *A. terreus* was studied by varying the incubation period of fermentation flasks from 0 to 18 days. The production of lovastatin (crude concentration) was increased till 7 days of fermentation. After that it was gradually decreased. The growth of fungal cell as indicated by the dry cell weight was also maximum during day 7. Therefore, an incubation period of 7 days was found to be the best for lovastatin production by *A. terreus*.

UV-Vis Spectrum Analysis

The spectrum analysis during biosynthesis of lovastatin was also carried out to confirm at which day the maximum amount of lovastatin synthesized by measuring the maximum absorbance at 238nm. It was found that at 7th day, maximum production of lovastatin was achieved (Figure 5).

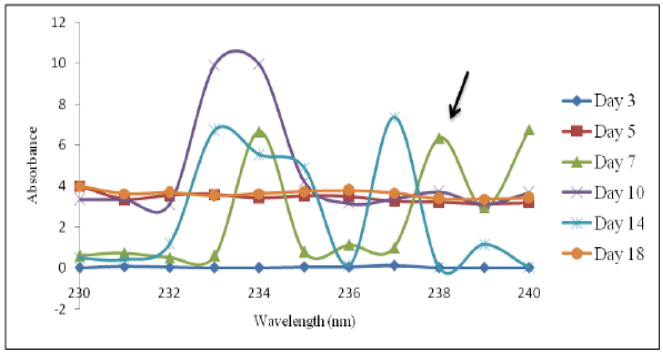

Figure 5: Spectrum analysis for different incubation periods

TLC study

The screening of lovastatin production by *A. terreus* NCIM 657 at different incubation periods was performed by TLC and presented in Figure 6 and Table 3. The crude lovastatin was purified and the analysis of TLC results showed same R_f as observed by standard lovastatin (0.52) at day 7. No synthesis of lovastatin was observed after the optimal incubation period (Table 3).

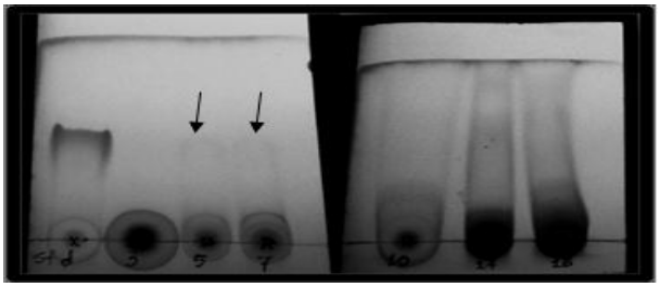

Figure 6: TLC of A. terreus NCIM 657 for different fermentation periods, against Standard Lovastatin

Table 3: Rf values of *A. terreus* NCIM 657 for different fermentation periods

Sample	Rf
Standard	0.52
Day 3	0.24
Day 5	0.51
Day 7	0.52
Day 10	Not detected
Day 14	Not detected
Day 18	Not detected

Bioassay of Lovastatin against *C. albicans and N. crassa*

The bioassay of lovastatin against *Candida albicans* and *Neurosporra crassa* cultures showed inhibition of growth of these indicators as reported by some studies and zone of inhibition also provides a criteria for lovastatin production. The results of bioassay against *C. albicans* and *N. crassa* revealed that maximum inhibition was observed at day 7 of fermentation process in both cases of indicators (Figure 7 and 8). A maximum zone of inhibition of 14.5 mm against *C. albicans* and 24 mm against *N. crassa*, respectively was observed at optimal incubation period of fermentation. Ferron *et al.* (2005) has also studied the zone of inhibition on plates of *C. albicans*. Prakash *et al.* (2014) also showed that *Saccharomyces cerevisiae* can also be used for the zone of inhibition study of lovastatin from *Aspergillus terreus*.

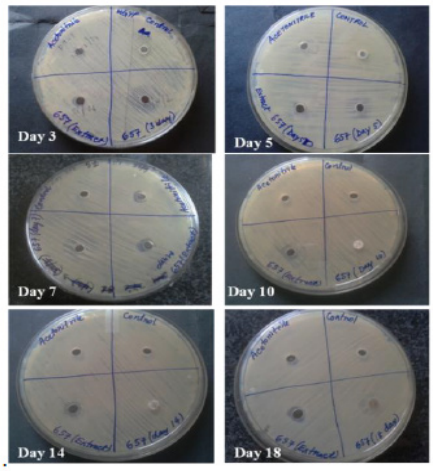

Figure 7: Bioassay of sample A. terreus NCIM 657 from different incubation period against C. albicans

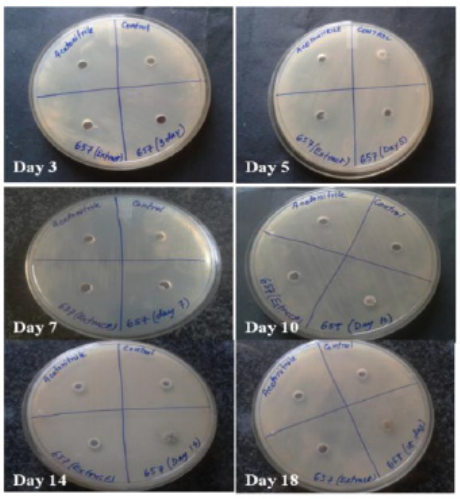

Figure 8: Bioassay of sample A. terreus NCIM 657 from different incubation period against N. crassa

Effects of different carbon sources on production of Lovastatin

Lovastatin productivity can be affected by different types of carbon sources used in the fermentation process. In some publications, lactose has been proven to be a better carbon source for Lovastatin. In our study, a number of media has been formulated for production of lovastatin from *A. terreus* NCIM 657 and the results of bioassay are presented in Figure 9. The TLC analysis of all the extracted and purified samples is also shown in the Figure 10. It has been observed that media II, IV and V were more suitable for production of lovastatin as compared to the medium containing glucose as main carbon source (Medium I). Starch (Medium IV) and the combination of Glucose, Lactose and Starch (Medium V) contributed the maximum yield of lovastatin. No lovastatin production was observed in Medium VI. ZhihuaJia *et al.* (2009) observed that glycerol containing culture media produced highest lovastatin titre (937.5± 12.5 mg/l), followed by the soluble starch containing production medium. Rf values of lovastatin for different media is presented in table 4; most of which correspond to standard lovastatin.

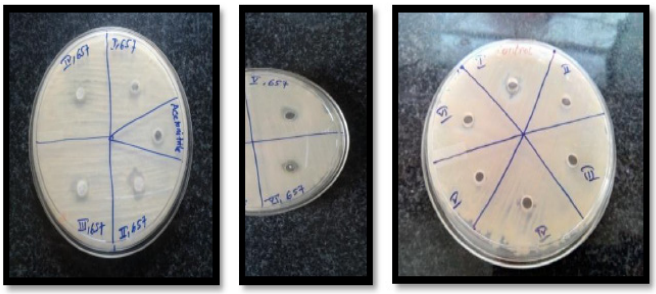

Figure 9: Bioassay of lovastatin produced in presence of different media against N. crassa.

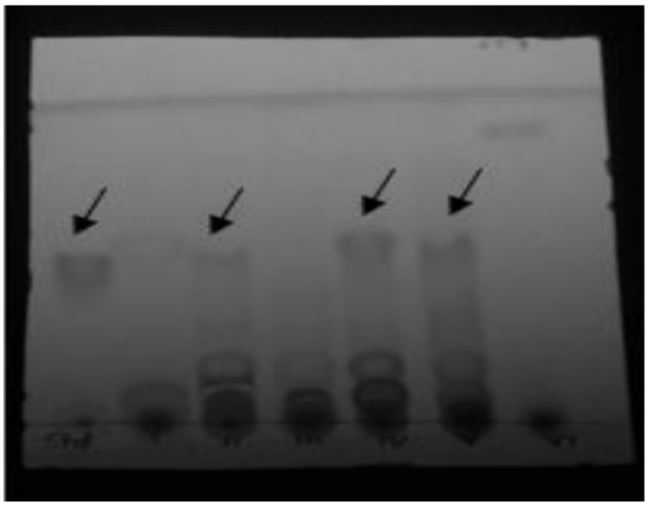

Figure 10: TLC of Samples from different carbon sources, against Standard Lovastatin for A. terreus NCIM 657

Table 4: Rf values of lovastatin for different media

Sample	Rf
Standard	0.52
Media I	0.54
Media II	0.52
Media III	0.50
Media IV	0.54
Media V	0.55
Media VI	0.73

FTIR analysis

The FTIR analysis of purified lovastatin from *Aspergillus terreus* NCIM 657 is shown in Figure 11 along with the FTIR of standard lovastatin. The spectrum showed a characteristics peak at 1721cm^{-1} which confirm the presence of lactone ring of statin. Other important peaks are 2962 cm^{-1}, 2860 cm^{-1}, 1455 cm^{-1}, 2314 cm^{-1} respectively present in standard lovastatin were also observed in the purified sample of the fermentation.

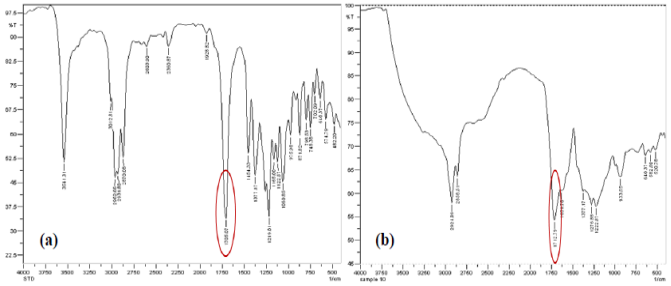

Figure 11: FTIR spectra of (a) Standard Lovastatin; (b) *A. terreus* NCIM 657

CONCLUSION:

In this present study, an attempt was made to optimize the fermentation of lovastatin using *Aspergillus terreus* NCIM 657 as producing organism. The concentration of lovastatin produced from the organism was 159 mg/L at a pH of 6.5 and temperature of 28 °C. The combination of carbon sources (Media V) which was not yet reported showed higher yield of lovastatin as compared to the glucose supplemented medium. FTIR analysis, TLC, bioassay and spectrum analysis confirmed the synthesis of secondary metabolite, lovastatin. The maximum lovastatin production was observed at 7 days of fermentation process under batch mode of cultivation.

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