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Production of Lovastatin from *Aspergillus terreus* (MTCC 1782) Under Solid State Fermentation

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ABSTRACT

Lovastatin ($C_{24}H_{36}O_5$) is a fungal secondary metabolite that inhibits conversion of 3-hydroxy-3- methylglutaryl coenzyme A (HMG CoA) to mevalonate in cholesterol biosynthesis. Lovastatin producing fungus *Aspergillus terreus* was grown in Solid State Fermentation (SSF) with various agro based wastes and to evaluate the suitable growth medium for maximum production of lovastatin. Lovastatin production by *Aspergillus terreus* and solid state fermentations (SSF) have been studied. To evaluate the ability to produce lovastatin various cultivation media and substrates have been used. The obtained data showed good lovastatin yield by *A. terreus MTCC1782* SSF. Five various types of bran have been tested as solid substrates for production of lovastatin in SSF - wheat bran, oat bran, maize bran, rice bran and mix of wheat and peanut bran. It has been observed that fermentation of wheat and *A. terreus 1782* on oat bran causes the highest lovastatin yield – **465.31 mg/lit** .

Keywords: Lovastatin *Aspergillus terreus* , solid state fermentation, production

INTRODUCTION

Lovastatin is a fungal secondary metabolite used for lowering blood cholesterol. It acts as an effective inhibitor of the enzyme hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase (mevalonate: NADP1 oxydoreductase, EC 1.1.1.34) that catalyzes the reduction of HMG-CoA to mevalonate during synthesis of cholesterol (Alberts *et al.*, 1980). It has been shown that lovastatin very competitively inhibits the reductase which decrease serum cholesterol levels by blocking cholesterol biosynthesis.

Lovastatin has a polyketide structure and is produced as a secondary metabolite by a variety of filamentous fungi such as *Monascus (M. ruber, M. purpureus, M. pilosus, M. anka), Penicillium (P. citrinum), Paecilomyces viridis*, and *Aspergillus (A. terreus)* (Manzoni *et al.*, 2002).

Commercial production of lovastatin is conventionally performed by liquid SmF using *A. terreus* mutants (Barrios-Gonzales *et al.*, 2010). To date, there are many publications focused on studies of cultivation regimes for producing statins (Bizukojc *et al.*, 2009). In the last years, SSF is becoming an alternative to SmF for generating many fungal products including statins. Comparative studies have shown that solid-state fermentation has advantages over SmF such as higher and faster yield, and less water need in up-stream processing which minimizes production expense (Holker *et al.*, 2004).

1. MATERIALS AND METHOD

Solid State Fermentation

Two grams of each substrate (Table1) was weighed and moisture content was maintain

70%. One milliliter of spore suspension (10^8spores) of *A. terreus* (MTCC1782) was added to the sterilized substrate and incubated at 28ºC[17].

Table 1: List of solid substrates (agro based materials) used for lovastatin production in *A. terreus*

Sl. No.	Substrate	Sl. No.	Substrate	Sl. No.	Substrate
1	Almond	22	Colocassia	43	Rice husk
2	Arecanut flower stem	23	Corn cob	44	Tapioca Sago
3	Arecanut seed	24	Corn kernel	45	Ragi bran
4	Ashgourd seed	25	Corn peel	46	Raisins
5	*Averrhoa bilimba* fruit	26	Cotton seed cake	47	Saw dust
6	Bamboo Rice husk	27	Cowpea	48	Sesame seeds
7	Banana flower	28	Date seed	49	Semolina
8	Banana flower bract	29	Dates (dried)	50	Soybean powder
9	Banana peel	30	Dates (fresh)	51	Spinach
10	Banana stem	31	Egg plant	52	Sprouted wheat
11	Barley	32	Flat Beans	53	Sugarcane bagasse
12	Beetroot	33	French Beans	54	Sunflower cake
13	Black gram	34	Gram husk	55	Spinach
14	Bread (white)	35	Green gram bran	56	Sweet potato
15	Bread (wheat)	36	Green Peas	57	Tamarind shell
16	Broken Red rice	37	Green Pigeon Peas	58	Tapioca peel
17	Cabbage	38	Ground nut cake	59	Tomato (sun dried)
18	Carrot	39	Ground nut shell	60	Urud dhal
19	Chick Peas	40	Guava	61	Wheat bran
20	Coconut cake	41	Jack fruit seeds	62	Wheat husk
21	Coffee husk	42	Jowar husk	63	Yam

1.1. Isolation of fungal culture from oyster mushroom bed

The contaminant fungi observed in oyster mushroom bed were isolated using standard microbiological techniques. The infected straw bits were surface sterilized with 0.1 per cent mercuric chloride solution for 30 sec. and washed with sterile distilled water for three times. They were then placed on PDA medium in sterile petridish and incubated at 30˚C for about 10 days. The culture was purified by single spore isolation technique and maintained on PDA slants and subculturing was done whenever necessary.

1.2. Isolation of fungal cultures from soil

Soil samples collected from paddy breeding station and s**u**saram Shrimp aq**ua** farm were also used for isolation of fungi. The fungal cultures were isolated by serial dilution technique using PDA medium. The isolated culture were purified by hyphal tip method and identified**.**

1.2.1. Single hyphal tip method of purification (Mundkur, 1959)

Plain agar medium containing 2 per cent agar-agar in tap water, after sterilization was poured into a sterile petri plate. After solidification, carefully lifted peripheral mycelial bits were observed for hyphal developments. Peripheral tip of the mycelial growth was taken from the plates and reinoculated to PDA medium. After proper documentation and coding, these purified colonies were transferred to PDA slants for further study.

1.3. Solid state fermentation

Lovastatin production by solid state fermentation was evaluated for the selected fungal isolates. A quantity of 70 g of substrate was taken in a 250 ml of conical flask and moistened with water to maintain the moisture at 40 per cent. The initial pH of the substrate was 5.0. The substrate was enriched with nutrient solution (Szakacs *et al.*, 1998). After draining the excess water, flasks were steam sterilized. Spores of the fungal isolates were inoculated to the flask. The content of the flask was stirred with sterile scalpel. After incubation for 10 days, at room temperature the contents were dried at 40˚C in an incubator. After the drying, the material was ground to fine powder in a porcelain pestle and mortar. The fine powder was used to estimate the lovastatin content using UV Spectrometry (Morovjan *et al.*, 1997).

1.4. Extraction of lovastatin in solid state fermentation

After 10 days of incubation the SSF material was ground into finely powdered material using sterile pestle and mortar. Each of 5 g of finely powdered SSF material were extracted with 150 ml of acetonitrile by shaking on a rotary shaker for 60 min at 220 rpm, then further centrifuged for 10 min at 3000 rpm.

1.5. Effect of substrates on lovastatin production

To find out the suitable substrate for maximum lovastatin production, substrates like wheat bran, rice bran, maize flour and sorghum grain were taken in the solid state fermentation process. The substrates were prepared by earlier described procedure (2.3) and inoculated with fungal spores and incubated for 15 days at room temperature. After 10 days of incubation the lovastatin yield was calculated by UV Spectrometry analysis (Morovjan *et al.*, 1997).

3.2.6 Lovastatin extraction:

At the end of 10 days of fermentation, the fermentation broth was acidified to pH 3.0 with 10% 1 N HCl. Then the acidified broth was extracted with equal volume of methanol under shaking condition (180 rpm) at 70°C for 2 hrs. The fungal biomass was separated by filtration using pre-weighed Whatman filter paper. The filtrates were subsequently centrifuged at 3000 g for 10 min and the organic phase was collected.

3.2.7 Quantitative analysis of lovastatin

UV spectroscopy

Ultraviolet absorption spectra were obtained with a UV- visible spectro photometer (shimadzu). A stock solution of lovastatin in methanol was prepared. Wave length scanning between 200- 400nm was performed. A standard curve from 0 to 1000 ug/ml was prepared. All measurements were performed in triplicate. The wave length at which the maximum absorption was observed and which was read the calibration curve was 238 nm. At this wave length there is no interference with methanol.

Procedure for quantitative analysis of lovastatin:

To the 1ml of organic phase 1% trifluoroacetic acid (10 ml) was added for lactonization process. Then the extract was concentrated at 80°C (without vacuum), to this add 10 ml of methanol for qualitative and quantitative estimation by UV Spectrometry. The concentration of lovastatin (µg/ml) present in the sample is obtained by plotting the O.D values on standard graph.

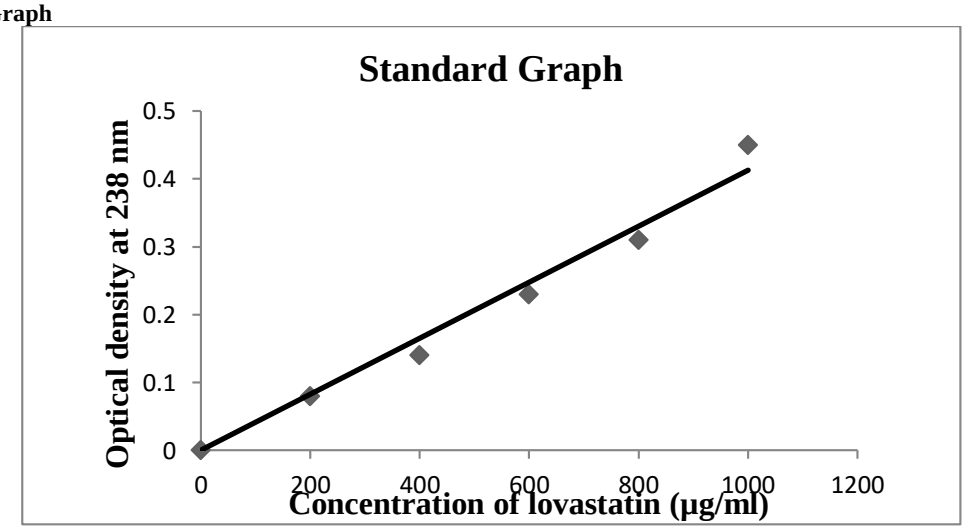
3.2.8 Standard graph:

99.9% pure lovastatin(lactone form) is dissolved in methanol to produce solutions of concentrations 1000µg, 800µg, 600µg, 400µg and 200µg/ml in different test tubes. Each test solution is made upto a volume of 10 ml with methanol and their absorbance is read spectrophotometrically at 238 nm.

Table 3.8 :Standard grap

Concentration (µg/ml)	O.D at 238 nm
200	0.08
400	0.14
600	0.23
800	0.31
1000	0.45

Figure 3.2 : Standard Graph



4.1 Optimization of process parameters

In order to improve the yield of lovastatin production, various parameters were studied and optimized. They include effect of fermentation time, effect of fermentation temperature, effect of pH, effect of inoculum volume, effect of inoculum age. Impact of carbon and nitrogen (both organic and inorganic) sources on the growth of *Aspergillus terreus* MTCC 1782 were also studied. The protocol adopted for the optimization of process parameters was to evaluate the effect of individual parameter at a time and to incorporate it at the standard level before optimizing the next parameter.

4.1.1 Optimization of fermentation time:

Optimization of fermentation time is carried out by inoculating 3ml of (6% v/v) inoculum(7 day culture) to 250 ml Erlenmeyer flasks, containing 50 ml of the sterilized medium-2 They were incubated for different fermentation times ranging from 24 to 240 hrs to obtain production profile of lovastatin. The flasks were analyzed for every 24 hrs.

4.1.2 Optimization of age of the inoculum:

To determine the optimum age of inoculum for the production of lovastatin by varying the age of inoculums from 3 to 10 days of 6% v/v inoculums is added in 250 ml Erlenmeyer flasks, containing 50 ml of the sterilized medium-2 and incubated at 32^0C. the flasks were analyzed after 192 hrs (10 days).

4.1.3 Optimization of inoculum concentration:

An experiment was carried out to optimize the size of inoculum for the production of lovastatin ranging from 2 - 8% of inoculum(6 day old culture) is added in 250 ml Erlenmeyer flasks, containing 50 ml of the sterilized medium-2 and incubated at 32^0C. the flasks were analyzed after 192 hrs (10 days).

4.1.4 Optimization of fermentation temperature

To determine the optimized temperature for lovastatin production, 5% v/v inoculum(6 day culture) is added in 250 ml Erlenmeyer flasks, containing 50 ml of the sterilized medium-2 and incubated at different fermentation temperatures 26,28,30,32,34 and 36^0C.The flasks were analyzed after 192 hrs (10 days)

4.1.5 Optimization of pH :

To determine the optimized pH for lovastatin production, 5% v/v inoculum(6 day culture) is added in 250 ml Erlenmeyer flasks, containing 50 ml of the sterilized medium-2 and pH is maintained with buffer solution (phosphate and acetate) incubated at different pH ranges of 3,4,5,6,7,and 8 at 32^0C, flasks were analyzed after 192 hrs (10 days).

4.1.6 Effect of nutrient source

4.1.6.1 Effect of different carbon sources

Different organic carbon sources (Maltose, Sucrose, Fructose, Glucose Lactose and Starch) were added to the media at 3% w/v concentration to each flask, and incubation parameters maintained were :

Inoculum age : 8 day old culture
Inoculum volume : 5%v/v
Temperature : 32°C
pH : 6.0
Flasks were analyzed after 144 hrs. (6days)

4.1.6.2 Effect of Glucose concentration:

To determine the optimum concentration of Glucose for lovastatin production Different concentrations ranging from 1- 5 %w/v were added to each flask, and incubation parameters maintained were:

Inoculum age : 8 day old culture
Inoculum volume : 5%v/v
Temperature : 32°C
pH : 6.0
Flasks were analyzed after 144 hrs. (6days)

4.1.6.3 Effect of different nitrogen sources

Various organic (peptone, corn step liquor, beef extract and yeast extract) and inorganic (sodium nitrate and ammonium sulfate) nitrogen sources are added to the fermentation media at 0.8% w/v concentration to each flask, and incubation parameters maintained were :

4.1.6.4 Effect of ammonium sulfate concentration

To determine the optimum concentration of ammonium sulfate for lovastatin production Different concentrations ranging from 0.1- 0.8 %w/v were added to each flask, and incubation partameters maintained were:

Inoculum age : 8 day old culture
Inoculum volume : 5%v/v
Temperature : 32°C
pH : 6.0
Flasks were analyzed after 144 hrs. (6days)

5. Results and Discussion

5.1 Screening of media

Two different fermentation media (Medium- 1 and Medium- 2) were screened for the production of lovastatin by *Aspergillus terreus* MTCC 1782 in shake flasks. It was found that fermentation media Medium- 2 was most suitable for the production of lovastatin. So, the culture medium Medium-2 was selected for further studies.

5.2 Effect of fermentation time:

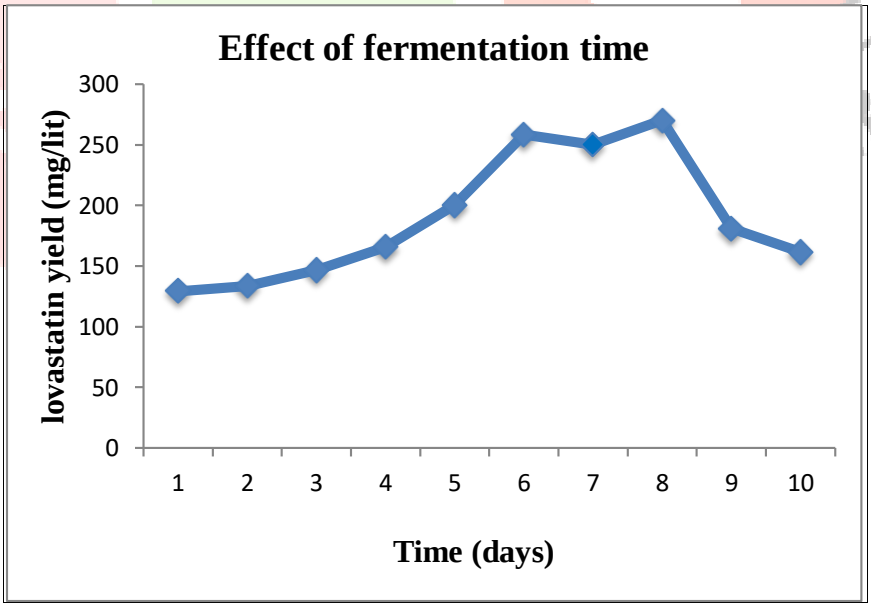
The effect of incubation period on the lovastatin production by *A. terreus* MTCC 1782 was studied by varying the incubation period of fermentation flasks from 24 to 240 hrs (1- 10 days). The production of lovastatin after 24 hrs of incubation was **130.21** mg/lit in the fermentation broth and increased with increasing incubation period reaching to the maximum value of **270.28** mg/lit after **192** hrs of incubation in the fermentation broth. It declined when further incubation was given to the fungal strain in the shake flasks.

Lopez *et al*., (2003) reported maximum lovastatin production with *Aspergillus terreus* was obtained on 150 hrs in their experiments under , solid state fermentation.

Table 5.1 : Effect of fermentation time

Incubation time (hrs)	Concentration (mg/lit)
24	130.21
48	134.52
72	147.53
96	176.86
120	210.31
144	235.36
168	250.23
192	**270.28**
216	260.80
240	221.57

Figure 5.1 : Effect of fermentation time



5.3 Effect of inoculum age

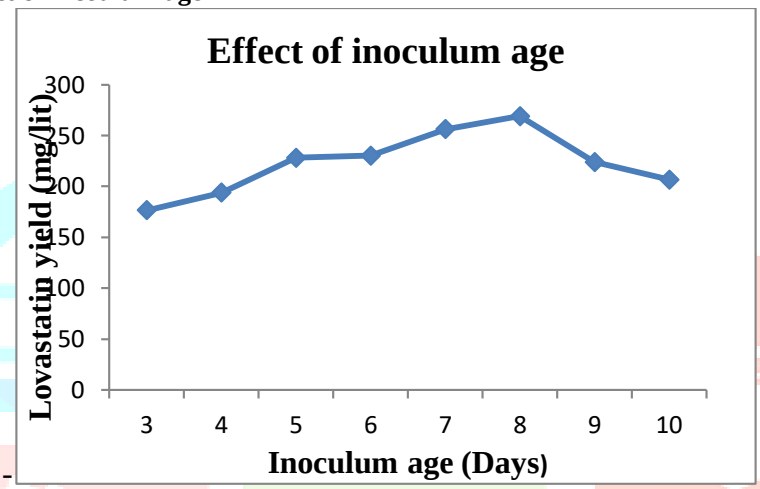
The effect of inoculum age on the production of lovastatin by *A. terreus* MTCC 1782 was studied. Different ages of the fungal vegetative inoculum ranging from 72 – 240 hrs were tested for lovastatin production. Maximum lovastatin production i.e., **269.01** mg/lit in fermentation broth was obtained using inoculum age of **8** days.

Arjumand ahmed *et al*., (2012) reported maximum lovastatin production with 8 days old culture of *Aspergillus terreus* under solid state fermentation ..

Table 5.2 : Effect of inoculum age

Inoculum age (Days)	Concentration (mg/lit)
3	175.58
4	196.72
5	220.16
6	230.09
7	250.10
8	**269.01**
9	221.86
10	200 .68

Figure 5.2 : Effect of incoulum age



5.4 Effect of inoculum concentration :

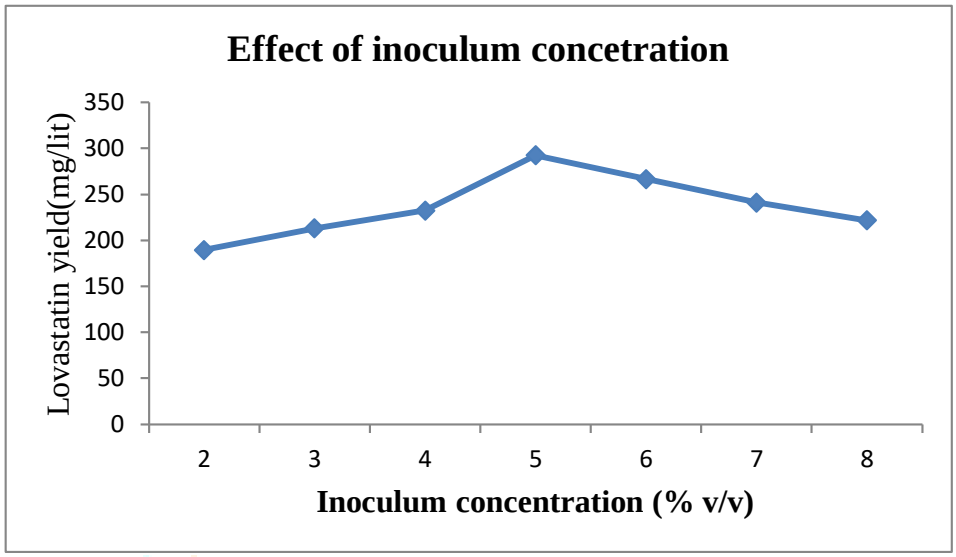
An experiment was carried out to optimize the size of inoculum ranging from 2–8% for the lovastatin production.The results showed that maximum lovastatin production was **299.88** mg/lit in the fermentation broth when **5%** (v/v) inoculum was used inoculate the fermentation flasks.

Hajjaj *et al.,* (2001) and Samiee *et al.,* (2003) reported maximum production of lovastatin using 6 %v/v of *Aspergillus terreus* solid state fermentation .

Table 5.3 : Effect of inoculum concentration

Inoculum concentration (%v/v)	Concentration (mg/lit)
2	177.41
3	211.01
4	232.44
5	**299.88**
6	269.87
7	251.06
8	221.72

Figure 5.3 : Effect of inoculum concentration



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5.5 Effect of fermntation temperature:

The effect of different incubation temperatures on the lovastatin production by *A. terreus* MTCC 1782 was studied by culturing the shake flasks in the temperatures ranging from 26 to **36°C,** From the results, it was observed that there was a gradual increase in lovastatin production when the incubation temperature was increased from **26°C** to **32°C**. The maximum lovastatin production, **311.27** mg/lit was observed at **30°C** in the fermentation broth respectively. The lovastatin production was gradually decreased with an increase of temperature from **30°C** to **36°C**.

The maximum production of lovastatin at **32°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. Arjumand ahmed *et al*., (2012)

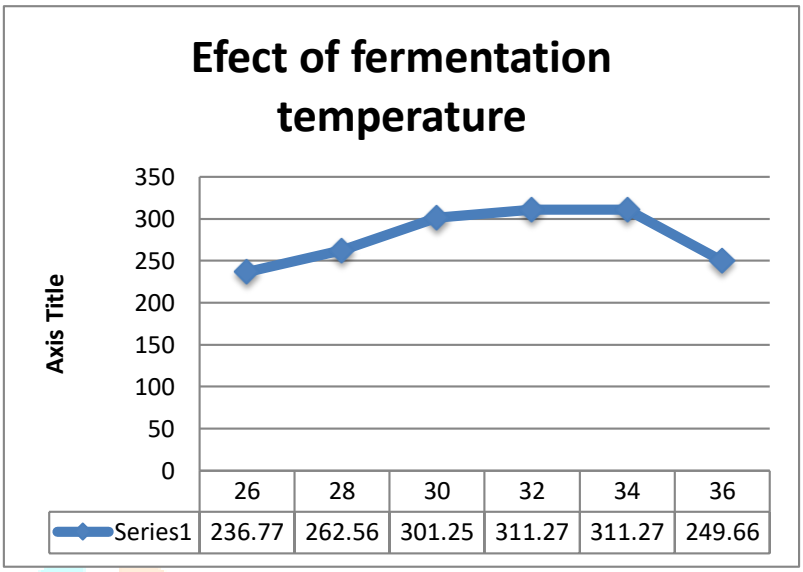
Samiee *et al.*, (2003), and Gupta *et al.*, (2007) reported maximum lovastatin production with *Aspergillus terreus* at a temperature of 32°C in their experiments under solid state fermentation.

Atalla *et al*.,(2008) reported maximum lovastatin production with *Aspergillus terreus at a* temperature of 32°C in their experiments under solid state fermentation.

Table 5.4 :Effect of fermentation temperature

Temperature (°C)	Concentration (mg/lit)
26	243.66
28	284.66
30	290.09
32	**311.27**
34	241.30
36	223.54

Figure 5.4: Effect of fermentation temperature



5.6 Effect of initial pH:

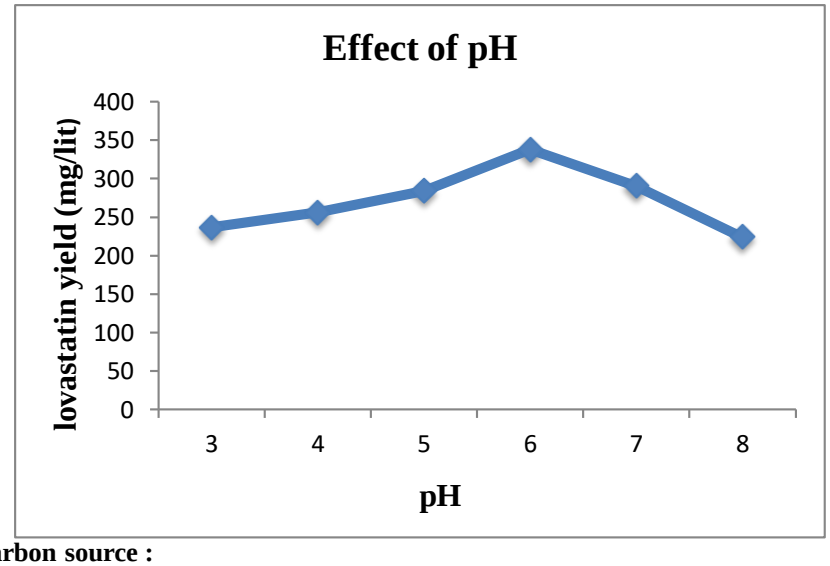
The effect of initial pH of culture medium on the production of lovastatin was studied by varying the initial pH of culture media from **3.0** to **8.0**. Lowest lovastatin production was observed at pH **3.0** i.e., **236.77** mg/lit in the fermentation broth. The production was started to increase at higher pH values and maximum lovastatin production i.e., **387.88** mg/lit in the fermentation broth was observed at **pH 6.0** and then it was decreased by increasing the initial pH above **6.0.** All the secondary metabolic activities normally occur at some specific pH and variation of pH during the fermentation process drastically affect them 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 (Kysilka, 1993).

Madan & Thind, (2000) reported maximum lovastatin production with *Aspergillus terreus* at pH 6.0 in their experiments under submerged fermentation.

Table 5.5: Effect of pH

pH	Concentration (mg/lit)
3	223.66
4	245.14
5	274.07
6	**387.88**
7	298.90
8	277.84

Figure 5.5 : Effect of pH



5.7 Screening of carbon source :

Different carbon sources including glucose, maltose, lactose, sucrose, fructose and starch were evaluated for the maximum lovastatin production by *A. terreus* MTCC 1782. The results showed that glucose gave maximum lovastatin productio i.e.,**352.10** mg/lit in fermentation broth. followed by lactose with a slight difference.

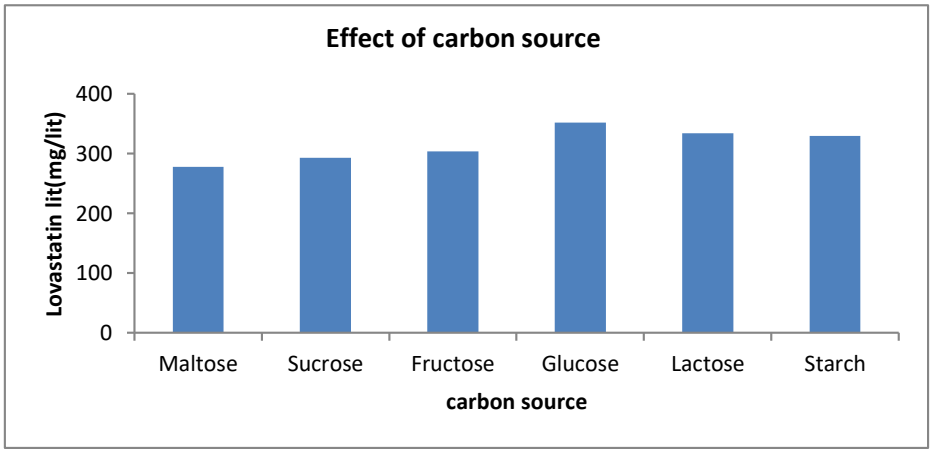
It is due to the fact that it is easily available carbon source and it oxidized very rapidly in the cells thus act as a readily available source of energy. Hajjaj *et al.*, (2001).

Hajjaj *et al.*, (2001) also Reported significant increase in lovastatin production using glucose as carbon source for *Aspergillus terreus* under submerged fermentation

Table 5.6 : Effect of carbon source

Carbon source (3 %w/v)	Lovastatin yield (mg/lit)
Maltose	223.31
Sucrose	289.95
Fructose	312.50
Glucose	**352.10**
Lactose	330.69
Starch	377.78

Figure 5.6: Effect of carbon source



5.8 Effect of glucose concentration:

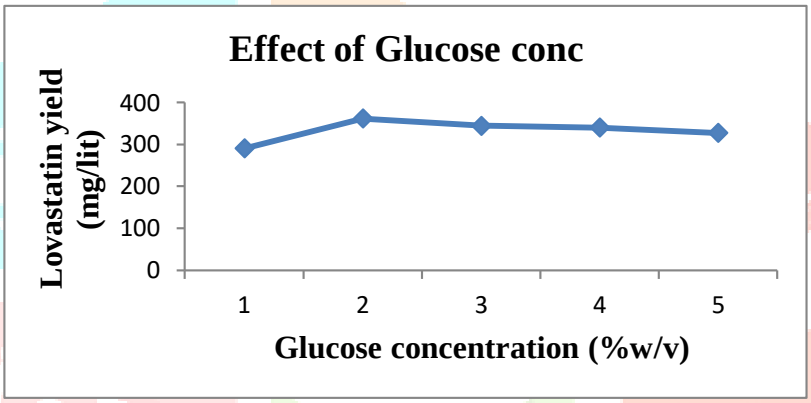
An experiment was carried out to optimize the concentration of **glucose 1-5 (%w/v)** in the culture medium. Yield of lovastatin was increased as the glucose concentration was increased and reached maximum **369.48** mg/lit at **2%** glucose concentration Therefore, **2%** glucose was selected as the most suitable concentration for the production of lovastatin by *A. terreus* MTCC 1782.

Arjumand ahmed *et al*., (2013) reported maximum lovastatin production yield obtained with 5% w/v of glucose as carbon source by *Aspergillus terreus* in their experiments under submerged fermentation.

Table 5.7 : Effect of glucose concentration

Concentration of glucose (%w/v)	Lovastatin yield (mg/lit)
1	291.66
2	**369.48**
3	340.22
4	329.83
5	338.94

Figure 5.7: Effect of glucose concentration



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5.9 Screening of nitrogen source :

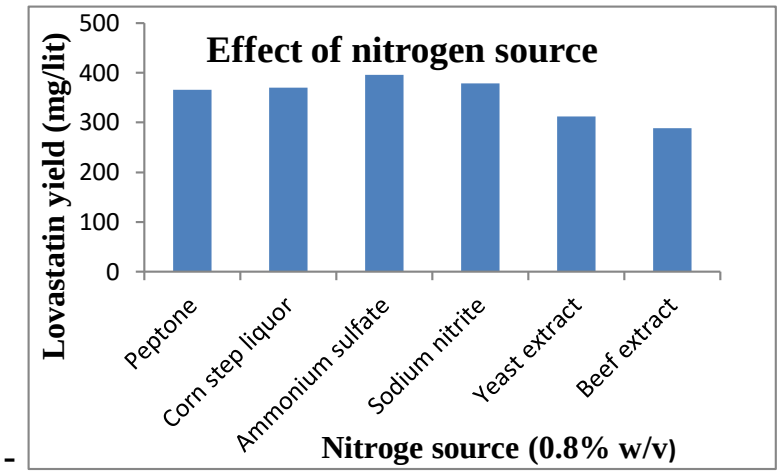
Different nitrogen sources i.e. organic and inorganic such as corn steep liquor, peptone, beef extract yeast extract, ammonium sulphate and sodium nitrite were evaluated for the lovastatin production by *A. terreus* MTCC 1782. Of all the sources, **ammonium sulfate** gave maximum lovastatin production i.e., **397.87** mg/lit in the fermentation broth.

This results accordance with lopez *et al*., (2003) results and reported as significant increase in lovastatin production using ammonium sulfate as inorganic nitrogen source for *Aspergillus terreus* under submerged fermentation.

Table5.8 : Effectof nitrogen source :

Nitrogen source (0.8 %w/v)	Lovastatin yield (mg/lit)
Peptone	332.89
Corn step liquor	379.09
Ammonium sulfate	**397.87**
Sodium nitrite	377.69
Yeast extract	351.90
Beef extract	238.55

Figure 5.8: Effect of nitrogen source :



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5.10 Effect of Ammonium sulfate concentration:

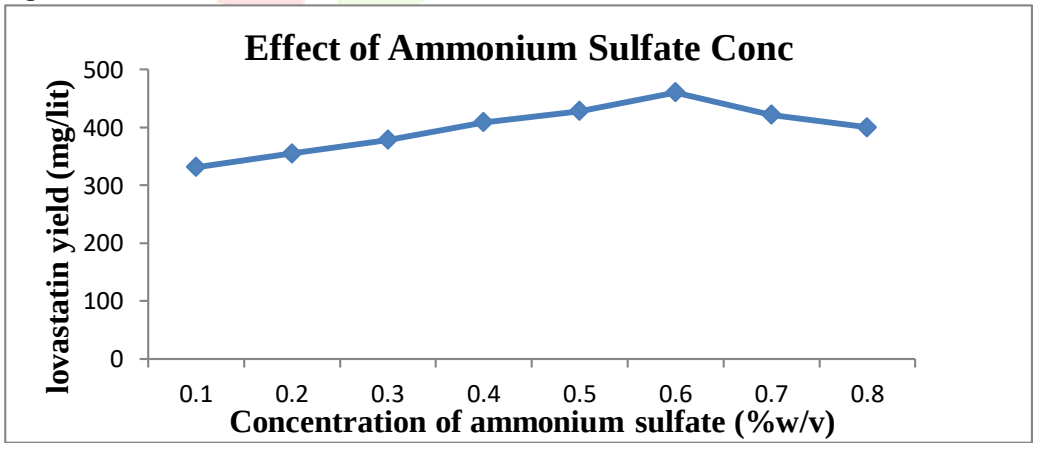
The amount of **ammonium sulfate (0.1-0.8%)** was further optimized for the production of lovastatin. and It was noted that ammonium sulfate at **0.6%** concentration gave maximum yield of lovastatin i.e., **465.31 mg/lit** in the fermentation broth.

Arjumand ahmed *et al*., (2013) reported maximum lovastatin production yield obtained with 0.3 %v/v of ammonium sulfate as nitrogen source by *Aspergillus terreus* in their experiments under submerged fermentation.

Table 5.9 : Effect of Ammonium sulfate concentration:

Concentration of ammonium sulfate (%w/v)	Lovastation yield (mg/lit)
0.1	337.21
0.2	355.96
0.3	388.89
0.4	428.81
0.5	441.06
0.6	**465.31**
0.7	431.63
0.8	400.12

Figure 5.9 :Effect of Ammonium sulfate concentration.



5.11 Optimized parameters

The lovastatin production by *Aspergillus terrus* MTCC 1782 under Solid State Fermentation (SSF) was carried out under optimized conditions. Which include:

Fermentation tme	:	**192** hrs (8 Days)
Inoculum age	:	**8** Day old Culture
Inoculum volume	:	5 (% v/v) spores
Temperature	:	**32**°C
pH	:	6
Carbon source	:	Glucose
Glucose concentration	:	2 (%w/v)
Nitrogen source	:	Ammonium sulfate
Ammonium sulfate concentration	:	0.6 (%w/v)

Optimization of all these factors resulted in the maximum production of lovastatin. **465.31 mg/lit**

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Temperature	:	**32**°C
pH	:	6
Carbon source	:	Glucose
Glucose concentration	:	2 (%w/v)
Nitrogen source	:	Ammonium sulfate
Ammonium sulfate concentration	:	0.6 (%w/v)

Optimization of all these factors resulted in the maximum production of lovastatin. **465.31 mg/lit**

6.1 SUMMARY

- In the present study, the production of lovastatin by *Aspergillus terreus* MTCC 1782 under submerged fermentation has been carried out.
- Medium- 2 gave a high lovastatin production in submerged fermentation.
- The physico-chemical process parameters of fermentation like incubation, temperature, pH, Inoculum volume and Inoculum age were optimized by single step -by-step optimization process. The nutritional supplementation study was carried out using some carbon, nitrogen sources to enrich the production medium and to enhance the lovastatin yield. The optimized conditions and maximum activities obtained are as follows.

- **Fermentation time**
  Maximum lovastatin yield of **260.36 mg/lit** obtained after **8** days of incubation.
- **Inoculum age**
  6 day old culture of Inoculum was observed to be the optimum for high yield of lovastatin **269.01 mg/lit** through submerged fermentation
- **Inoculum volume**
  Optimum Inoculum concentration of 5% **(v/v)** is observed suitable for the production of lovastatin of **299.88 mg/lit.**
- **Fermentation temperature**
  Maximum lovastatin yield of **311.25 mg/lit** was obtained at **32ºC** on **8** days of incubation
- **Initial pH**
  Maximum lovastatin yield of **336.78mg/lit** was obtained at **pH 6**.
- **Carbon source**
  Addition of Glucose **(2% w/v)** as carbon source showed maximum Lovastatin yield of **397.87 mg/lit.**
- **Nitrogen source**
  Addition of ammonium sulfate **(0.6% w/v)** as nitrogen source showed maximum lovastatin yield of **465.31 mg/lit.**

6.2 CONCLUSION:

The strain initially showed **164.57 mg/lit** of lovastatin production. After optimization of the physical and nutritional conditions, *Aspergillus terreus* MTCC 1782 was capable of producing about 3 fold increased lovastatin i.e., **465.31 mg/lit** in the fermentation broth. The strain holds a promise for scale up production of lovastatin under submerged fermentation after a comprehensive study.

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