Screening of different fungi for production of lovastatin

Riya Dhar, Gourab Basu Choudhury, Vinod Kumar Nigam*
Department of Bio-Engineering, Birla Institute of Technology, Mesra, Ranchi-835215, Jharkhand, India

Research Article

Article Info
Recived on:23/04/2015
Accepted on: 15/05/2015
Published on: 25/05/2015

QR Code for mobile

Literati
Open Access

ABSTRACT :

Statin based compounds are widely accepted as cholesterol lowering agent. These are classified as natural, synthetic or semi synthetic derivatives of natural statins. Lovastatin is one the natural statin that acts as a competitive inhibitor of 3-hydroxy 3-methylglutaryl Coenzyme-A reductase (HMG Co-A reductase), the rate limiting enzyme of the cholesterol biosynthesis. Various fugal isolates are capable of producing lovastatin as secondary metabolite by polyketide synthesis pathway. In the present study, seven wild fungi were screened for lovastatin production by bioassay method against *C. albicans* and *N. crassa*. The results showed that, *Aspergillus terreus* NCIM 657 produced the maximum lovastatin (0.989 mg/ml) in the screening work through submerged fermentation. Other fungal isolates recorded lower yield of lovastatin. Further study with spectrum analysis at 238 nm and thin layer chromatography confirmed lovastatin synthesis by two more fungi i.e. *Aspergillus parasiticus* NCIM 898 and *Phoma exigua* NCIM 1237.

KEY WORDS: *Aspergillus terreus,* Bioassay, HMG Co-A reductase, Lovastatin

INTRODUCTION:

Statin based compounds has an extremely high therapeutic value and other biological activities. Natural statins like lovastatin and compactin are produced by the fermentation, while there are a number of other semisynthetic statins produced by biotransformation. Simvastatin, the second leading statin in the market is also derivative of lovastatin [1]. Lovastatin ($C_{24}H_{36}O_5$, Mevinolin, Monacolin K) is a fungal polyketide based secondary metabolite widely used as a prominent drug in hypercholesterolemia. It stops cholesterol synthesis by competitively inhibiting the rate limiting enzyme 3-hydroxy 3-methylglutaryl Coenzyme-A reductase (HMG Co-A reductase) of the cholesterol synthetic pathway [2, 3].

This compound decrease the level of cholesterol concentration in blood; particularly bad cholesterol (low density lipoprotein, LDL); while slightly increasing the level of good cholesterol (high-density lipoprotein, HDL), thus, preventing plaque build-up inside the arteries and hence, it decreases the risk of heart attack or stroke or cardiovascular diseases [4]. Besides these the recent studies also showed that statins can be used for treatment of alzheimer's disease [5], multiple sclerosis [6], renal disease treatment [7], bone maturation [8] and to some extent in treatment of cancer [9]. Lactone form of lovastatin has comparatively higher activity as compared to acid form.

Fungi are important sources for the production of several pharmaceutical compounds. They produce a large variety of compounds mainly through the polyketide biosynthesis pathway. Statins, among these classes of fungal metabolites have become the focus of great attention due to their ability to influence the *de novo* synthesis of endogenous cholesterol [10]. Of many statin molecules, lovastatin and mevastatin are natural, while other statins like rosuvastatin, simvastatin, pravastatin, fluvastatin, atrovastin, cerivastatin, are produced semi-synthetically from lovastatin [11]. Lovastatin is produced as a secondary metabolite by a variety of filamentous fungi. The production starts from the acetate units in head to tail function to form two polyketide chains [12]. Commercially it is produced from *Aspergillus terreus*[13] but, it can also be synthesized from various fungal strains like *Penicillium sp.* [12], *Monascus ruber* [14], *Monascus purpereus, A. paraciticus, Acremonium chrysogenum, Trichoderma viridae, Pleurotus sp.* [10]. Commercial production of lovastatin by batch fermentation using *A. terreus* has been investigated extensively [15]. In submerged fermentation, the yield of lovastatin is proportional to the biomass accumulated. The lovastatin can also be produced by solid state fermentation, which uses sub-

doi: 10.15272/ajbps.v5i44.699

Corresponding author:
Dr. Vinod Kumar Nigam
Department of Bio-Engineering, Birla Institute of Technology, Mesra, Ranchi-835215, Jharkhand, India
Email: vknigam@bitmesra.ac.in

Conflict of interest: Authors reported none

strates that require lesser processing with better yield than submerged fermentation [16].

Screening of different fungi from various natural resources confirmed that some of the molds are able to secrete lovastatin in liquid culturing conditions. Certain marine actinomycetes were also reported as lovastatin producers [17]. Besides these, many edible fungi of higher basidomycetes are capable of producing cholesterol lowering drug; especially *Pleurotus sp.* which is used for production of single cell protein. [18]

Many studies have been performed for the production of lovastatin from various environmental sources and optimized process for maximum yield, the present work also highlighted the synthesis of lovastatin from different wild type fungal strains. The production was confirmed by various analytical methods and finally a comparative study on the production process has been reported.

MATERIALS AND METHODS:

Organisms and Fermentation Condition:

Different fungal strains including some wood rot fungi was obtained from NCIM (National Collection of Industrial Microbiology, Pune, India) as mentioned in Table 1. These cultures were used screened for the lovastatin production by submerged fermentation. The stock culture was periodically grown on PDA (Potato Dextrose Agar) plates for 6-7 days at 28 °C. The production of lovastatin was carried out in shake flask under agitation speed of 170-180 rpm for 7-8 days at 28^{0}C in the production media at pH 6.5 as described by Gupta *et al.* 2009 (Table 2). The 50 ml of medium was prepared in conical flask and it was sterilized at 121 °C for 20 minutes. 6 mm block each from freshly grown PDA culture plates was aseptically transferred to the production media and production of lovastatin was monitored. At the end of production of lovastatin pH of broth, crude lovastatin concentration as well as zone of inhibition by product against indicators was recorded.

Name of Fungi	NCIM No.
Aspergillus terreus	654
Aspergillus terreus	657
Aspergillus terreus	660
Aspergillus parasiticus	898
Pleurotus sajor caju	1133
Pleurotus ostreatus	1200
Phoma exigua	1237

Table 1: Different fungi for lovastatin production

Sl. No.	Constituents	Concentration
1	Glucose	45 g/L
2	Monosodium Glutamate	12.6 g/L
3	KH_2PO_4	4 g/L
4	K_2HPO4	5 g/L
5	$FeSO_4.7H_2O$	0.2 g/L
6	$MnSO_4.4H_2O$	0.1 g/L
7	$MgSO_4.7H_2O$	0.1 g/L
8	$ZnSO_4.7H_2O$	0.2 g/L
9	$CaCl_2.2H_2O$	20 mg/l
10	$CuCl_2.2H_2O$	5 mg/l
11	H_3BO_3	11 mg/l
12	$(NH_4)_2MoO_4$	5 mg/l

Table 2: Composition of production media (19)

Extraction of lovastatin:

At the end of fermentation process, broth was acidified to pH 3.0 with concentrated H_2SO_4/ HCl for lactonization purpose [2]. It was then extracted with two volume of ethyl acetate and kept on rotary shaker at 170 rpm for 2 h at 28 °C. The samples were subsequently centrifuged at 10,000 rpm for 15 minutes for separation of organic phase, which was dried and dissolved in 2 ml of acetonitrile for estimation of lovastatin.

Bioassay:

The method for screening of lovastatin producing molds by bioassay was adopted from Kumar *et al.* (2000) and Vilches *et al.* (2005) [20, 21] using *Neurospora crassa* and *Candida albicans* as indicator organisms. In bioassay method, a clear inhibition zone around the indicator organisms is observed and the diameter of the zone of inhibition is proportional to the concentration of the lovastatin in the samples. Indicator cultures, *Neurospora crassa* (NCIM 863) and *Candida albicans* (NCIM 3102) were maintained in PDA and MGYP plates respectively at 28 ^{0}C for 24 - 48 h. The concentration of standard lovastatin with respect to diameter of zone of inhibition against indicator organisms were performed and used for calculating the presence of lovastatin in the crude samples.

UV/Vis spectrometer analysis :

The estimation of lovastatin was also calculated spectrophotmetrically by Double beam UV/Vis spectrometer (LABTRONICS Model LT 2900). The standard and extracted sample exhibited a peak at 238 nm during the analysis of spectrum. The standard plot and spectrum analysis of different concentrations of lovastatin was performed for identification of potential producers of lovastatin from the selected fungi.

Thin Layer Chromatography:

Thin layer chromatography (TLC) was also used for the identification of lovastatin sample from the select-

ed molds. For this, aluminum TLC sheet pre coated with Silica Gel F_{254} were used. The plate was initially activated by heating in micro-oven for 1- 2 min. A quantity of 20 µl of the crude extract along with the known concentration of standard lovastatin was spotted above 15 mm of the solvent in the chromatographic chamber. The chromatograms were developed in a solvent system (mobile phase) of dichloromethane: ethyl acetate in the ratio of 70:30 (v/v). The visualization of the developed spots was marked under UV chamber (254nm) and Rf values were recorded and compared.

RESULTS:

All the seven fungal strains that were grown under submerged culture conditions for production of lovastatin were evaluated by bioassay method against indicators *Candida albicans* and *Neurosporra crassa*, by measuring the absorbance at a wavelength 238 nm and finally by TLC respectively. Based on the observation three fungi were selected as potential statin producer.

Screening of lovastatin producers by bioassay method:

The screening of potential lovastatin producing molds were carried out by measuring the zone of inhibition against indicators and the concentration of lovastatin was calculated using standard plot of pure lovastatin. Different concentrations of standard lovastatin (0.2 - 2.0 mg/ml) were prepared and zone of inhibition against *C. albicans* and *N. crassa* were performed by well method (Figure 1). The standard plot of zone of inhibitions against different concentrations of lovastatin is presented in Figure 2 (a) and (b).

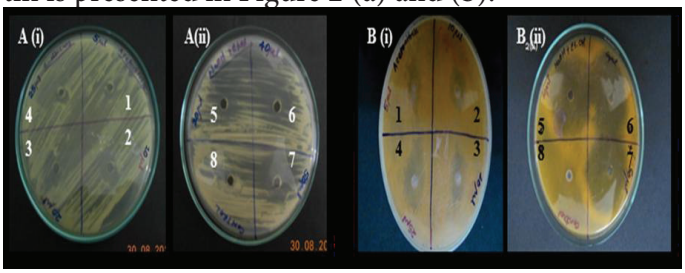

Figure 1: Bioassay of standard lovastatin against *C. albicans* (A- i & ii) and *N. crassa* (B- i & ii) at different concentrations (mg/ml); (1) 0.2; (2) 0.4; (3) 0.8; (4) 1.0; (5) 1.2 ; (6) 1.6; (7) 2.0 and (8) Control

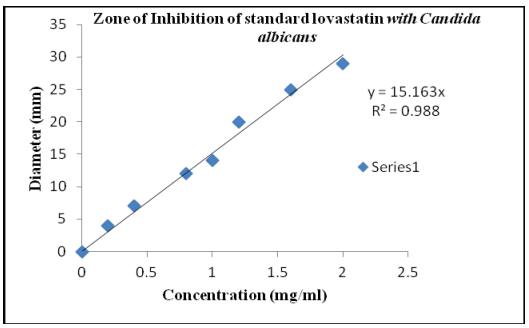

Figure 2 (a): Standard plot of lovastatin vs zone of inhibition against *C. albicans*

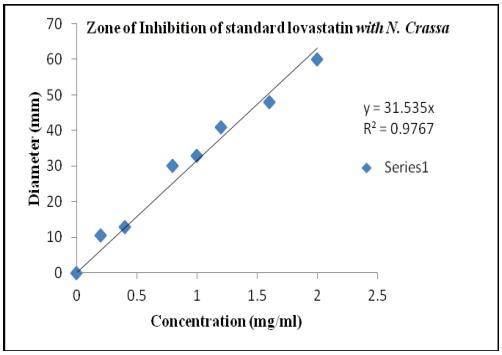

Figure 2 (b): Standard plot of lovastatin vs zone of inhibition against *N. crassa*

The zone of inhibition by the extracted lovastatin from different fungi against indicators is shown in Figure 3 followed by concentration of lovastatin from the standard plot in Table 3

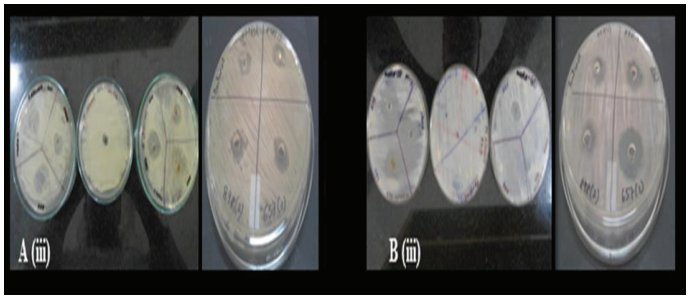

Figure 3: Bioassay of samples from different fungal strains *Aspergillus terreus* NCIM 660, *Aspergillus terreus* 654, *Aspergillus parasiticum* 898, *Phoma exigua* 1237, *Pleurotus ostreatus* 1200, *Aspergillus terreus* 657 against *C. albicans* (A iii) and *N. crassa* (B iii)

Strains (NCIM No.)	Zone of inhibition against *C. albicans* (mm)	Crude Lovastatin Concentration (mg/ml)	Zone of inhibition against *N. crassa* (mm)	Crude Lovastatin Concentration (mg/ml)
Aspergillus terreus (654)	10	0.659	21	0.665
Aspergillus terreus (657)	15	0.989	30	0.951
Aspergillus terreus (660)	7	0.462	16	0.507
Aspergillus parasiticum (898)	13	0.875	27	0.856
Pleurotus sajor caju (1133)	--	--	--	--
Pleurotus ostreatus (1200)	9	0.593	19	0.603
Phoma exigua (1237)	12	0.791	24	0.761

Table 3: Concentrations of crude lovastatin produced from different fungi

Based on the diameter of zone of inhibition, it has been observed that maximum lovastatin production was found with *Aspergillus terreus* NCIM 657 (0.989 mg/ml), followed by *Aspergillus parasiticum* NCIM 898 (0.87mg/ml) and *Poma exigua* NCIM 1237 (0.79 mg/ml). All other molds produced the lovastatin in low concentration. There was no trace of lovastatin in the extracted sample obtained from the broth of *Pleurotus sajor caju* using the selected medium, hence no zone of inhibition was found.

Confirmation and quantification of lovastatin by λmax:

The confirmation of the presence of lovastatin in the extracted samples was also carried out by measuring the absorbance at 238 nm using spectrophotometer and the concentration of crude product by different fungi was calculated against standard plot of lovastatin (Figure 4). Figure 5 showed the absorbance of different concentrations of lovastatin in the range of 200 to 300nm.

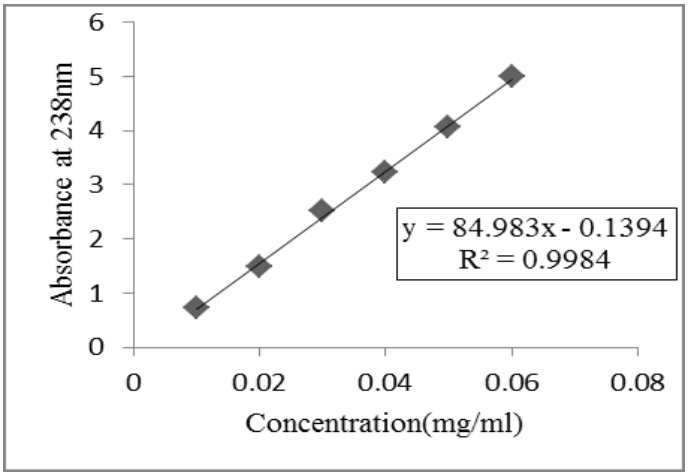

Figure 4: Standard plot of lovastatin at 238 nm

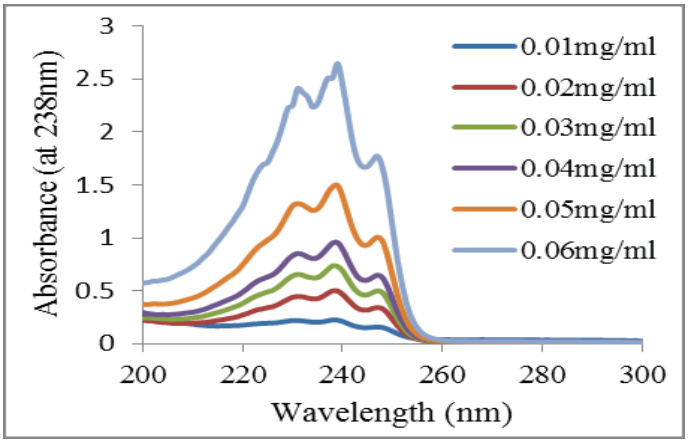

Figure 5: Spectrum analysis of lovastatin

The concentration of crude lovastatin produced from three fungi as calculated from the standard plot is shown in Table 4.

Name of fungi (NCIM No.)	Concentration of lovastatin (mg/ml)
Aspergillus terreus (657)	0.159
Aspergillus parasiticum (898)	0.157
Phoma exigua (1237)	0.062

Table 4: Concentration of crude lovastatin

Based on the standard plot and spectrum analysis, it has been inferred that three fungi namely *Aspergillus terreus* NCIM 657, *Aspergillus parasiticum* NCIM 898 and *Phoma exigua* NCIM 1237 are potential lovastatin producing fungi. The spectrum analysis of samples is presented in Figure 6.

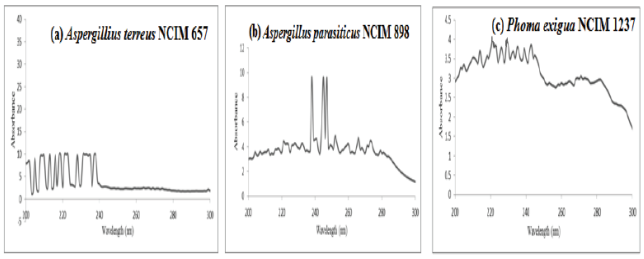

Fig 6: Spectrum analysis of crude lovastatin from (a) *Aspergillus terreus* NCIM 657 (b) *Aspergillus parasiticus* NCIM 898 and (c) *Phoma exigua* NCIM 1237

Identification of lovastatin by Thin Layer Chromatography:

The lovastatin was also identified by thin layer chromatography (Figure 7) and R_f value was calculated and compared with the authentic lovastatin as shown in Table 5

Strain (NCIM No.)	R_f
Aspergillus terreus (657)	0.53
Aspergillus parasiticum (898)	0.52
Phoma exigua (1237)	0.53
Pleurotus sajor caju (1133)	Not detected
Standard Lovastatin (L+A)	0.52

Table 5: TLC of different samples (NCIM)

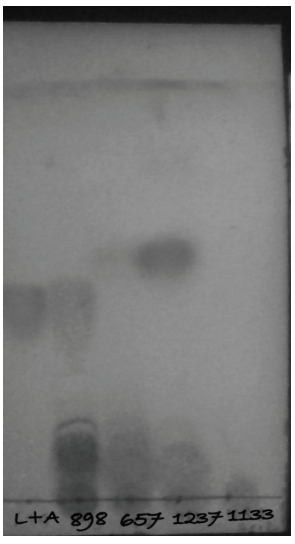

Figure 7: TLC chromatogram of standard as well lovastatin produced from different molds

The kinetic study of production of lovastatin from three molds is shown in Figure 8. It has been observed that maximum biomass was formed with *A. parasiticus* (NCIM 898) though the concentration of lovastatin was low compared to *A. terreus* (NCIM 657). Maximum glucose was utilized by *A. terreus* and least by *Phoma* which produced less concentration of lovastatin at 7 days of fermentation process.

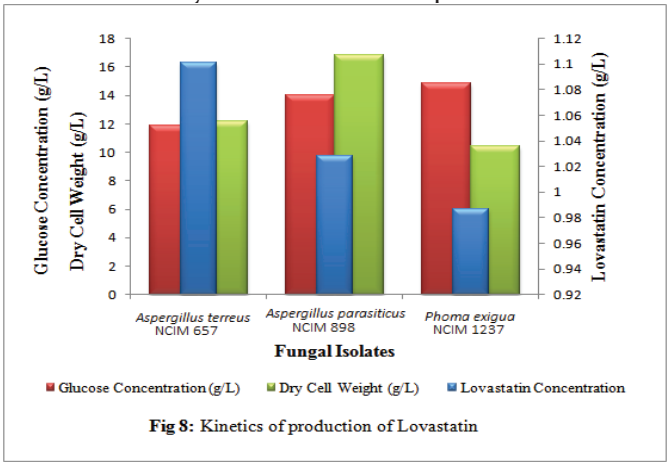

Fig 8: Kinetics of production of Lovastatin

DISCUSSION:

The screening methods as Bioassy and spectrum analysis applied in this study permitted isolation of lovastatin producer from different wild type of the fungi in a significantly shorter period as compared to the other existing methods. UV spectrum analysis provides confirmation of synthesized product as well as an idea of concentration of the product. These methods of screening were less expensive and required less labour. Results obtained from these two methods can further be validated by HPLC which is more accurate but time consuming. Ignoring small modifications in fermentation conditions (media compositions), the current study differs from the ones by Gunde *et al.* (1973) [22] and Shindia (1997) [23] where lovastatin was extracted using methanol as solvent followed by TLC and HPLC analysis while ethyl acetate was used for extraction of lovastatin in the present study. Ethyl acetate assisted to increase the sensitivity of detection owing to good solubility of lovastatin and possibility of concentrating the organic solvent. Extraction of acidified product with ethyl acetate followed by dissolving of the dried product in acetonitrile leaves the lovastatin in the lactone form. This protocol enhanced recovery of product unlike in the case of methanol extraction, where a significant amount of lovastatin remains in open hydroxyl acid form, posing difficulties in detection.

In a previous study by Samiee *et al.* [2] a large number of microbes was screened for production of lovastatin where *Aspergillus terreus* was identified as the best lovastatin producing fungus (production level up to 55 mg/l) among other lovastatin producers. Gunde *et al.* [22] also identified certain microorganism for synthesis of lovastatin by thin layer chromatography and high pressure liquid chromatography. The species screened as lovastatin producers were *Aspergillus terreus* (up to100 mg/l), *Paecilomyces variotii* and *Pythium ultimum*. Other microbes with low concentration of lovastatin (1 mg/l to 4.5 mg/l) were *Aspergillus flavus, Aspergillus niger, Aspergillus repens, Aspergillus versicolor, Penicillium variable, Pleospora herbarum* and *Trichoderma viridae* repectively.

Shindia [23] reported lovastatin production from *Aspergillus oryzae, Aspergillus terreus, Doratomyces stemonitis, Paecilomyces variotii, Penicillium citrinum, Penicillium chrysogenum, Scopolariopsis brevicaulis* and *Trichoderma viridae* etc with *Aspergillus terreus* as best producer.

In the present study, out of seven fungi, three were observed as lovastatin producers; *Aspergillus terreus* NCIM 657 (0.989 mg/ml), *Aspergillus parasiticus* NCIM 898 (0.875 mg/ml) and *Phoma exigua* NCIM 1237 (0.791 mg/ml) respectively in the selected medium. Further optimization of medium constituents and other process parameters may enhance the production of lovastatin and its yield.

ACKNOWLEDGEMENTS:

Authors duly acknowledge the financial assistance received from All India Council for Technical Education, Government of India, New Delhi, [(Ref. No: 20/AICTE/RIFD/RPS(Policy-1), 24/2012-13] for carrying out this work and Centre of Excellence, Department of Bio-Engineering for infrastructure and instrumentation facilities under TEQIP, Phase II (Ref No NPIU/TEQIP II/ FIN/31/158; 16th April 2013).

REFERENCES:

1.Javier BG, Roxana UM. Biotechnological production and applicatio ns of statins. Appl Microbiol Biotechnol 2010; 85 : 869– 883

2.Samiee SM, Moazami N, Haghighi S, Mohseni FA, Mirdamadi S, Bakhtiari MR. Screening of lovastatin production by filamentous fungi. Irani Biomed J. 2003; 7: 29-33

3.Nigam VK, Dhar R, Agarwal A, Khandelwal AK, Mohan MK, Vidyarthi AS, Ghosh P. Studies on production of fungal secondary metabolite lovastatin . Int. J. Adv. Res. 2014; 2: 978-986

4.The Expert Panel. Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Arch. Intern. Med. 1998; 148: 36–69

5.Eckert GP, Wood WG, Muller WE. Statins: Drugs for Alzheimer's disease? J Neural Transm. 2005; 112: 1057-71.

6.Zamvil SS, Steinman L. Cholesterol-lowering statins possess anti-inflammatory activity that might be useful for treatment of MS. Neurology. 2002; 59: 970-1

7.Buemi M, Senator M, Corica F. Satins and progressive renal disease. Med Res Rev. 2002; 22: 76-84.

8.Garrett IR, Gutierrez GE, Rossini G, Nyman J, McCluskey B, Flores A, Mundy GR. Locally delivered lovastatin nanoparticles enhance fracture healing in rats. J. Ortho. Res. 2005; 25: 1351-1357.

9.Tandon V, Bano G, Khajuria V, Parihar A, Gupta S. Pleiotropic effects

of statins. Ind J Pharmacol. 2005; 37: 77-85.
10.Nidhiya KA, Sathya E, Nitya M. Extraction and purification of lovastatin from non-aflatoxigenic strains of Aspergillus flavus. Int J Bio Pharma Res. 2012; 4: 916- 921
11.Jonathan AT. Lovastatin and beyond: the history of the HMG-CoA reductase inhibitors. Nature review. 2003; 2:517-526.
12.Endo A. Compactin (ML236B) and related compounds as potential cholesterol-lowering agents that inhibit HMG-CoA reductase. J Med Chem. 1985; 28:401–405.
13.Alberts AW, Chen J, Kuron G, Hunt V, Huff J, Hoffman C. Mevinolin: a highly potent competitive inhibitor of hydroxymethyl glutaryl coenzyme A reductase and cholesterol lowering agent. Proc Natl Acad Sci USA. 1980; 77:3957-61.
14.Juzlova, P, Martinkova L, Kren V. Secondary metabolites of the fungus Monascus: a review. J. Ind. Microbiol. 1996; 16: 163-170.0
15.Manzoni M, Rollini M. Biosynthesis and biotechnological production of statins by filamentous fungi and application of cholesterol lowering drugs. Appl Microbial Biotechnol. 2002; 58: 555-564
16.Xu BJ, Wang QJ, Jia XQ, Sung CK. Enhanced Lovastatin Production by Solid State Fermentaion of Monascus ruber.Biotechnol Biopro Engg. 2005 10:78-84
17.SreeDevi K, Venkateswara RJ, Lakshmi NM, SaiKrishna K. Isolation and screening of lovastatin producing Aspergillus terreus fungal strains from soil samples. Int J Pharm Technol. 2011; 3: 2772-2782.
18.Wasser SP, Reshetnikov SV Process for producing, methods and compositions of cholesterol lowering agents from higher basidiomycetes mushrooms. US Patent. 2002; 6: 372 462
19.Gupta K, Mishra PK, Srivastava P. Enhanced continuous production of lovastatin using pellets and siran supported growth of Aspergillus terreus in an Airlift reactor. Biotechnology and Bioprocess Engineering. 2009; 14: 207-212
20.Kumar MS, Kumar PM, Sarnaik HM, Sadhukhan AK. A rapid technique for screening of lovastatin producing strains of Aspergillius terreus by agar plug and Neurospora crassa bioassay. J Microbiol Methods. 2000; 40:99 - 104.
21.Vilches FMA, Casas Lo´pez JL, Sa´nchez PJA, Ferna´ndez SJM, Chisti Y. Rapid screening of Aspergillus terreus mutants for overproduction of lovastatin. World J Microb Biot. 2005; 21: 123–125.
22.Gunde CN, Friedrich J, Cimerman A, Benicki N. Screening fungi for the production of an inhibitor of HMG CoA reductase: Production of mevinolin by the fungi o f the genus Pleurotus. FEMS Microbiol. Lett. 1973; 111:203-206.
23. Shindia, AA. Mevinolin production by some fungi. Folia Microbiol. 1997; 42:477-480.

Cite this article as:

Riya Dhar, Gourab Basu Choudhury, Vinod Kumar Nigam. Screening of different fungi for production of lovastatin. Asian Journal of Biomedical and Pharmaceutical Sciences, 5(44), 2015, 24-29.