JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2007. Vol. **1**(2), p. 295-299

Isolation, Screening and Lovastatin Production by Wild Strains of *Aspergillus terreus*

V.L. Maheshwari* and R.H. Patil[1]

[1]Department of Biochemistry, School of Life Sciences,
North Maharashtra University, Jalgaon - 425 001, India.
[2]R.C. Patel Arts, Commerce and Science College, Shirpur - 425 405, India.

(Received: 02 September 2007; accepted: 14 October 2007)

Studies were undertaken to isolate and screen the lovastatin producing fungi from the North Maharashtra region. Out of 30 isolated fungi 11 were exhibited zone of inhibition against *Candida albicans*. Of all the 11 strains investigated *Aspergillus terreus*PM3 exhibited highest zone of inhibition against *Candida albicans* and yielded 90 mg/l of lovastatin at 10 days in submerged cultures. Employing whole soybean flour medium *A.terreus* PM3 yielded 90 mg of lovastatin per lit. at 10 days. It was observed that diameter of zone of inhibition in bioassay was directly proportional to yield of lovastatin. Medium optimization experiments with *A. terreus* PM3 indicated that lactose and defatted soybean flour were best carbon and nitrogen sources respectively.

Keywords: *Lovastatin, Aspergillus terreus, Candida albicans, submerged cultures*.

Hypercholesterolemia is considered as important risk factors in atherosclerosis and coronary heart diseases (CHDs) (Dallas TX, 2001). Since more than two third of total body cholesterol is synthesized *de novo,* inhibition of the cholesterol biosynthesis is considered as important way to control cholesterol level (Alberts et *al,* 1980). Statins a class of fungal secondary metabolites, competitively inhibit the 3- hydroxyl-3-methylglutaryl coenzyme-A reductase (HMG-CoA reductase), the rate limiting step in the cholesterol biosynthesis by significantly lowering the blood cholesterol level in humans and animals. (Endo *et al.,*1976). Lovastatin also restricts the growth of tumor cells by inhibiting isoprenoid biosynthesis. Mevastatin was the first statin to be discovered as secondary metabolite of *Penicillium citrinum* (Endo *et al.,* 1979). Lovastatin was the first statin approved by FDA as a cholesterol lowering drug. Many organisms such as *Monascus rubber,Trichoderma, Penicillium* species, *Paecelomyces* species were known to produce lovastatin. Commercially the lovastatin is produced by batch cultures of *Aspergillus terreus* (Endo *et al.,* 1986.). Lovastatin is the prodrug, the inactive lactone, which is hydrolyzed to the corresponding beta hydroxyl acid form. The hypocholesterolemic activity of the beta hydroxyl acid form of lovastatin is due to its structural similarity to HMG-CoA, which is the natural substrate of the enzyme. Lovastatin is also known to decrease cholesterol in hepatocytes by increasing HDL (High Density Lipoprotein) and decreasing LDL (Low Density Lipoprotein). Lovastatin has also been reported to increase the number of LDL- specific membrane receptors which is important in cholesterol metabolism. Lovastatin is generally

* To whom all correspondence should be addressed.
Fax: +91-257-2258403
E-mail: vlmaheshwari@hotmail.com

produced using batch fermentations. Attempts have also been made for solid state fermentation. Fed batch fermentations were found to superior over batch cultures. (Kumar *et al.,* 2000). The lactone form of the lovastatin is inactive and water insoluble, while β-hydroxy acid form is active and water soluble. In fermentation broths 90% lovastatin exist in the later form. β-hydroxy acid form of lovastatin exhibits antifungal activity. Lovastatin inhibited the growth of *Candida albicans* at the concentration 0.4-1.0 micrograms. The antifungal activity of β-hydroxy acid form of the lovastatin was exploited using *Candida albicans* bioassay plates to evaluate lovastatin producing ability of the isolates (Kumar *et al.,* 2000).

In the present work attempts were made to isolate new potential producers of the lovastatin and to study lovastatin production by submerged culture at shake flask level.

MATERIAL AND METHODS

Isolation and screening of microorganisms

30 fungal strains were isolated form the 35 soil samples collected from the different regions of the North Maharashtra, India. All strains were maintained on the Czapek Dox agar slants at 28^0C. Identification was carried on the basis of morphological character as per Raper and Fennel. *Candida albicans* ATCC 4561 obtained from NCIM; Pune was maintained on PDA slants and used for bioassay plates (Kumar *et al.,* 2000).

100 ml medium of the following composition was used (lactose 60 g/ l, soybean meal 25 g/ l, corn steep liquor 5 g/ l, KH_2PO_4 2 g/ l, K_2HPO_4 1 g/ l, $MgSO_4$.7H2O 0.5 g/ l, $FeSO_4.4H_2O$ 1 g/ l, NaNO3 0.5 g/ l, $MnSO_4.7H_2O$ 0.5 g/ l, Agar15 g/ l and p^H 7.0) was sterilized and poured in to the presterilized flat bottomed 16 cm glass Petri plates (Borosil, India) and allowed to solidify. The agar plugs were prepared using sterile 10 mm diameter cork borer. Three agar plugs are then transferred to each glass slide (3 x 1 inch) in the sterile Petri plate containing Whatmann filter paper no 1. The spores collected from the 10 day old slant of isolates were scrubbed in sterile 2% tween 80 using wire loop and spore suspension was obtained. The agar plugs were then inoculated by holding the plug in sterile forcep and deeped in spore suspension at each surface and placed on the surface of the glass slide. To prevent drying of agar plugs humidity was maintained by adding 5 ml sterile distilled water to Whatmann filter paper and the Petri dishes were incubated in humidity chamber at 28^0C for 4 days. After incubation, agar plugs were transferred to screw capped test tube containing 2 ml ethyl acetate and agar plugs were macerated with sterile glass rod and tubes were kept for 3 hours (Kumar *et al.,* 2000).The resulting suspension was centrifuged and solids were removed by filtration, followed by centrifugation. *Candida albicans* was grown for 4 days on PDA plate. The cell no. of *Candida albicans* cells was optimized to 2 x 10^2 cells/ml. 0.1 ml of the 24 hr old culture of *Candida albicans* was spread over the surface of agar plate using sterile glass spreader. Wells were prepared by using 6mm diameter sterile cork borer and 0.5 ml extracted sample is added in each well in laminar air flow unit. 0.5 ml of the ethyl acetate and 0.5ml of β-hydroxy acid form of lovastaton was also added as a control and standard. Plates were kept in refrigerator for 15 minutes for diffusion and incubated for 48 hrs at 37^0C and zone of inhibition was observed.

Inoculation and culture conditions

The isolates showing highest zone of inhibition in bioassay, against *Candida albicans* were selected and maintained on the PDA (Potato Dextrose Agar) agar. After inoculation from the original slant, plates were incubated at 28°C for 5 days. A suspension of spores was obtained by scrubbing the plate with 5 ml of sterile aqueous solution of 2% tween 80. The spore concentration was optimized to 10^6 spores/ml by using newbors haemocytometer. Seed was developed by inoculating 5 ml spore suspension in 250 ml Erlenmeyer flask containing lactose 60 g/ l, soybean meal 25 g/ l, KH_2PO_4 2 g/ l, K_2HPO_4 1 g/ l, $MgSO_4$.7H2O 0.5 g/ l, $FeSO_4.4H_2O$ 1 g/ l, NaNO3 0.5 g/ l and $MnSO_4.7H_2O$ 0.5 g/ l. After developing sufficient biomass 8% of inoculam was transferred to production medium containing lactose 60 g/ l, soybean meal 25 g/ l, corn steep liquor 5 g/ l, KH_2PO_4 2 g/ l,, K_2HPO_4 1 g/ l,, $MgSO_4$.7H2O 0.5 g/ l, $FeSO_4.4H_2O$ 1, g/ l, NaNO3 0.5 g/ l and $MnSO_4.7H_2O$ 0.5 g/ l. The p^H of the medium was maintained to 7.0 (after sterilization). All fermentations were carried out

at 28 ^{0}C and 200 rpm for 10 days on rotary shaker (Steelmate Novatech, India) in 500ml shake flask contaning 150 ml of production medium (Manzoni *et al.,* 2002).

Analytical methods

Biomass and lovastatin recovery

After each 48 hour interval 20 ml sample was withdrawn and dry weight of the biomass was determined by centrifugation. Cell debris and Insoluble particles were removed and the clear solution was filtered through 0.45µm membrane filter. Residual sugar were determined spectrophotometrically (Shimadzu, Japan) using DNSA method (Dubos *et al.,* 1956). The fermentation broth obtained by filtration was acidified to p^H 3 with 0.1 N HCL and flask was kept on rotary shaker for 1 hour at room temperature. Extraction was carried out with 1:1 ethyl acetate in separating funnel and organic phase was collected. The extracts were dried and concentrated in a spray dryer and subjected for analyses.

Lovastatin analysis

Lovastatin was measured as its β-hydroxyacid form by high performance liquid chromatography (HPLC) of the biomass free broth. The lovastatin is synthesized in its β-hydroxyacid form in fermentation broth. The β- hydroxy form of the lovastatin elutes earlier than its lactone form. The purified fermented broth is diluted five times with acetonitrile and water (1:1). The standard for HPLC analysis was prepared by converting lactone form of lovastatin to β-hydroxy acid form by dissolving 10 mg. of standard lovastatin powder to 0.1 N NaOH and ethanol.(1:1), heating at 50^{0}C for 20 minutes and neutralizing with 0.1 N HCL. The resulting lovastatin stock solution containing 1 mg/ml lovastatin was maintained at 5^{0}C. HPLC was performed on Jupiter phenomenix 250 x 4.60 mm 4 U column with LC 20AT liquid chromatograph (Shimadzu, Japan) equipped with Shimadzu SOD – M20A diode array detector. The 20µl sample was injected and chromatograph was developed with the mobile phase 1:1 mixture of acetonitrile and distilled water. The flow rate was 2 ml/ minute. The detection wavelength was 238 nm (Fredrich *et al.,* 1995).

Chemicals and reagents

The pharmaceutical grade lovastatin was generous gift from Ranbaxy Laboratories, India. HPLC grade ethyl acetate and acetonitrile was supplied from Qualigens fine chem.

RESULTS AND DISCUSSION

In the initial part of the study, isolation and screening of the lovastatin producing strains was carried out using *Candida albicans* bioassay method. The objective of this study was to isolate the new potent producers of the lovastatin from the local region. In this study, 30 fungal strains were isolated, out of those 11 were found positive at the bioassay level. Of 11 strains of *Aspergillus terreus* PM3, PM6, and PM11 exhibited 17, 14 and 16 mm zone diameter respectively. The bioassay of *Candida albicans* showed uniform growth in bioassay plates with distinct zones of inhibition against pure β -hydroxy acid of lovastatin and agar plug extract. Disks with only ethyl acetate did not showed any zone of inhibition in any of three isolates. The diameter of zone of inhibition was directly proportional to the amount of lovastatin produced by the respective strain. *Aspergillus terreus* ATCC 20542 shown highest zone of inhibition and maximum lovastatin titer (Table 1).

To confirm the lovastatin producing ability of the isolated strains, fermentations were carried out at shake flask level. The isolates PM3, PM6 and PM11produced lovastatin with lesser yield than the commercially used strain *Aspergillus terreus* ATCC 20542 (Table 1). All further fermentations were conducted by employing *Aspergillus terreus* PM3, the high yielding strain. The highest lovastatin titer was obtained in batch cultures when spherical mycelial pellets were used as inoculam at aeration 200 rpm. After assessing which strain produce high titer of lovastatin, we turned our attention to the time course of lovastatin fermentation by *Aspergillus terreus* PM3. Lovastatin production was monitored after each 48 hours by HPLC. The *Aspergillus terreus* fermentation profile is shown in the Fig. 1.The highest lovastatin concentration 90mg/l in batch cultures was obtained in 10 days. The residual sugar concentration was not reduced significantly during the first two days of the fermentation. The biomass concentration has increased gradually from day 3 and maximum

Table 1. Lovastatin production by different isolates of *Aspergillus terreus*

S. No.	*Aspergillus terreus* strain	Zone diameter (mm)	Lovastatin titre mg/l against *C.albicans*
1.	*Aspergillus terreus* PM3	17	90
2.	*Aspergillus terreus* PM6	13	54
3.	*Aspergillus terreus* PM11	15	70
4.	*Aspergillus terreus* ATCC 20542	21	150

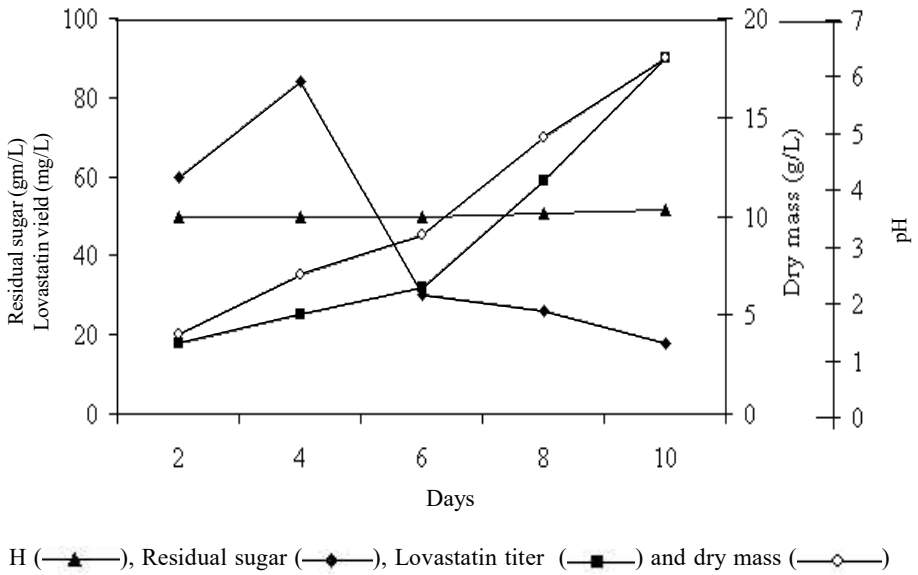

Fig. 1. Lovastatin fermentation profile of *Aspergillus terreus* PM3

amount of biomass is obtained at 10 days. The peak of the mycelial biomass was 22g/L. The p^H monitoring revealed that the p^H slightly becomes alkaline at the end of fermentation (7.2). The residual sugar concentration remains 18g/l at the end of fermentation. The morphology of mycelia during the course of fermentation has profound effect on lovastatin production in batch fermentation. At least in some secondary metabolite fermentation, pelleted growth has yielded higher than filamentous form of the fungi. The composition of the production media also plays the important role in maintaining the morphology, length and size of the hyphae. The filamentous growth of mycelia is usually develops when simple and rapidly metabolizing carbon sources are used. Use of some complex biopolymers restricts the filamentous growth of fungus and induces pellet formation, which improves the oxygen and mass transfer of the fermentation and thus ultimately increases the yield of the product (Data not shown).

Conclusion

The *Candida albicans* bioassay method can be used as a rapid screening method for isolating the lovastatin producing fungi from the natural sources. The highest lovastatin production 90mg/l was obtained in 10 days with *Aspergillus terreus* PM3 in submerged culture at p^H 7.O and aeration 200 rpm. It may be noted that lactose as a slowly metabolizing carbon source induces pellet formation and thus improves lovastatin yield.

ACKNOWLEDGEMENT

Authors are thankful to Dr. D. R. Patil for providing laboratory facilities and Prof. Ulhas Patil for valuable discussion on data representation.

REFERENCES

1. Dallas, T.X. *Heart and stroke statistical Update* American heart association. American heart association. 2001.
2. Alberts, A.W., 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: (39)**.57-61.
3. Endo, A., Kuroda, M., Tasujita, Y. ML-236 A, ML-236 B and ML- 236 C, new inhibitors of cholestrogenesis produced by *Penicillium citrinum*. *J. Antibiot.,* 1976; **29**:1346-8.
4. Endo, A. Monacolin K. A new hypocholesterolemic agent produced by *Monascus* species. *J Antibiot.* Tokyo, 1979; **32:** 852-4.
5. Endo, A. Productivity of monacolin K (mevinolin) in the genus *Monascus*. *J. Ferment. Eng.* 1986; **64**: 509–12.
6. Kumar, M.S., Jana, S.K., Senthil, V., Shashanka, S., Kumar, S.V., Sadhukhan. A.K. Repeated fed batch process for improving lovastatin production. Process .*Biochem.* 2000; **36**: 363-8.
7. Endo, A., Hausami, K., Yamada, A., Shimoda, R., Hiroshi, T. The synthesis of compactin (ML-236 B) and monacolin K in fungi. *J. Antibiot.* 1986; **39**: 1609-16.
8. Kumar, M.S., Sadhukhan, A,.K., Kumar., P.M., Sarnaik., H, M. A rapid technique for screening of lovastatin-producing strains of Aspergillus *terreus* by agar plug and *Neurospora crassa* bioassay. *J Microbiol. Methods.* 2000; **40** 99-5.
9. Manzoni, M., Rollini, M. Biosynthesis and biotechnological production of statins by filamentous fungi and application of these cholesterol lowering drugs. *Appl. Microbiol. Biotechnol.* 2002; **58**: 555-64.
10. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A, Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 1956; **28** 350-6.
11. Friedrich, J., Zuzek, M., Bencina, M., Cimerman, A., Strancar, A., Radez, I., High-performance liquid chromatographic analysis of mevinolin as mevinolinic acid in fermentation broths. *J. Chromatogr.* A. 1995; **704**; 363-4