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ISOLATION AND SCREENING OF LOVASTATIN PRODUCING *ASPERGILLUS TERREUS* FUNGAL STRAINS FROM SOIL SAMPLES

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Abstract:

In the present study various strains of *Aspergillus terreus* cultures were isolated from soils of different regions of Andhra Pradesh, India and were identified through cultural and microscopic characteristics and further screened for the production of lovastatin through agar plug assay method. Of the selected fungal isolates, KSVL-SUCP-75 (ONG-II) exhibited the maximum zone of inhibition against *Neurospora crassa* in bioassay. Lovastatin production was rapidly confirmed through the laboratory analytical techniques TLC and UV spectroscopy followed by the final confirmation through HPLC and IR Spectroscopy. *Aspergillus terreus* fungal strain (KSVL-SUCP-75) was further characterised by MTCC Chandigarh.

Key words: *Aspergillus terreus*, Bioassay, Lovastatin, TLC and, UV, IR.

Introduction:

Hypercholesterolemia is the proven risk factor in coronary artery diseases and statins are considered as a class of potent anti-hypercholesterolemic drugs[1,2] . Statins act by competitively inhibiting the enzyme 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, a rate limiting enzyme in cholesterol biosynthesis.[3, 4]. All natural statins possess a common polyketide portion, a hexahydro naphthalene ring system to which are attached different side chains.

Lovastatin ($C_{24}H_{36}O_5$) is a natural statin produced as a secondary metabolite by the various soil dwelling filamentous fungi viz *Aspergillus terreus* [2,5-10] *Monascus ruber*[11,12], *Penicillium citrinum*[2] and *Pleurotus*

sps.[14]Lovastatin isolated from *Aspergillus terreus* was the first statin to be approved by FDA in 1987 for therapeutic use.[4, 14] Aspergillus *terreus* is still the most commonly used producer of this wonder drug[15].

The agar plug extracts showing zone of inhibition in bioassay were rapidly confirmed by simple inexpensive analytical and techniques, TLC and UV spectroscopy for the presence of lovastatin. This was followed by further HPLC and IR spectroscopic confirmation of this secondary metabolite in fermentation broth.

Materials and Methods:

Isolation and Characterization of Soil Fungal Isolates:

In the present work soil samples collected from different regions of Andhra Pradesh, India were used for isolation of *Aspergillus terreus* fungal strains using Seifert's medium following standard microbial techniques[16].The soil fungal isolates were further maintained on PDA slants and characterized based on their colony morphology and microscopic observations[17]. *Neurospora crassa* FGSC 4200 (NCIM Pune) was used as the test organism in bioassay study.

Screening of *Aspergillus terreus* fungal isolates for Lovastatin production using agar-plug method[18]

Soyabean meal medium was used for screening and the composition is as follows (g/L): Sucrose: 50; Soyabean meal: 20; K_2HPO_4: 1; $NaNO_3$:1; $MgSO_4.7H_2O$: 0.5, Agar; 20g and pH 6.5. Medium was transferred aseptically into sterile petri plates. Agar plugs were prepared and carefully transferred onto a sterile glass slide placed in a petri plate containing circular whatman filter paper. To prevent the agar plugs from drying, whatman filter paper was moistened by spraying with sterile distilled water and further the plates were maintained in humid conditions during incubation. Spores of 10 days old cultures were used to inoculate onto the surface of the agar plugs. The inoculated plugs were incubated at 28^0C for 5 days.

Extraction of Lovastatin from agar plugs and *Neurospora crassa* Bioassay:

After 5 days of incubation the agar plugs were transferred into screw capped test tubes and was macerated with ethyl acetate. Spores of *Neurospora crassa* FGSC-4200 grown on Sabourad dextrose agar (SDA) slants for 10 days were used in the bioassay. 0.85% sterile saline containing 0.2% Tween 80 was used to harvest the spores. Sterile molten SDA medium maintained at 45^oC was seeded with 0.4 - 0.5 X 10^8 spores and poured into a flat bottomed pre-sterilized petri plate. After solidification of SDA medium, wells were made using a sterile borer of

8mm diameter and 50µl of the extract was loaded into the wells, simultaneously placing the standard lovastatin drug of known concentration. in each plate. Ethyl acetate was used as control. Plates were allowed for pre-diffusion and then incubated at 28^{o}C for 16-18 hrs. (Incubation beyond this specified period resulted in overgrowth of the test organism and thus the boundaries of inhibition zone could not be measured clearly). After incubation, zone of inhibition was measured.

TLC and U.V Spectrophotometric analysis of agar plug extracts:

Agar plug extracts exhibiting zone of inhibition against *Neurospora crassa* were further rapidly confirmed through TLC method.[13] TLC plates were spotted with of the acidified, concentrated extract in triplicate along with the standard sample. These plates were eluted with the solvent system Dichloromethane:Ethyl acetate (70:30).

The other solvent systems used for elution are:

1. Toluene : Ethanol (80:20)
2. Ethyl Acetate: Hexane: Acetic Acid (70:30:6)
3. Dichloromethane: Acetic Acid (85:15)

These eluted plates were exposed initially to U.V light and then to Iodine vapours to visualize the separated spots. R_f values were calculated and compared the values of agar plug extract samples obtained with that of standard samples. The separated spots on TLC plates exhibiting similar R_f values in both sample and standard were further scrapped and transferred carefully into sampling vials. To this was added the solvent ethyl acetate and filtered. Filtrate was scanned between 200-400nm in UV Spectrophotometer (Shimadzu) for determining the λ_{max}. The agar plug extracts showing λ_{max} at 238 nm were further estimated quantitatively according to the method described by K. Lingappa et al., (2004)[9].

Submerged fermentation, confirmation of Lovastatin by HPLC and IR spectroscopy:

The soil fungal isolate confirmed to have produced the maximum amount of lovastatin was further subjected to submerged fermentation using soyabean-meal screening medium (excluding agar). 50 ml of the medium was inoculated with 10 days old spore suspension. The flasks were incubated on a rotary shaker at 28^{o}C. After

incubation for a period of 7 days, P^H of the fermentation broth was adjusted between 2- 3 using hydrochloric acid.

An equal volume of ethyl acetate was added to the fermentation broth and kept on a rotary shaker for 2 hours for extraction of lovastatin. Thus separated ethyl acetate extract was washed with 5% Sodium Bicarbonate for 4-5 times, and with distilled water.[21] Thus resulted comparatively pure ethyl acetate extract was analysed through HPLC and IR for further confirmation of lovastatin.

HPLC method of Analysis: The prepared standard and the sample were analysed for lovastatin by high performance liquid chromatography (HPLC) in lactone form. For the preparation of the sample 20ml of the ethyl acetate extract was taken and dried under vacuum at 45^{o}C. To the residue an equal volume of the acetonitrile was added. The resultant was filtered using 0.45µm filter and further used for HPLC analysis.[20,21].

HPLC was carried out by employing the following chromatographic condition.

1. Column specifications: C18 column (Hypersil), with dimensions of 150x4.6 mm, 5 µm.
2. Mobile phase - Acetonitrile: 0.1% O-Phosphoric acid (60:40 v/v)
3. Sample injection volume: 20µl
4. Flow rate: 1.0 ml/min
5. Ambient temperature: 25^{O}c.
6. λmax: 238nm

A standard lovastatin sample was prepared by dissolving lovastatin pure drug (Dr.Reddy's Laboratories Ltd, Hyderabad.) in acetonitrile.

I.R Spectroscopic Determination:

Sample preparation for I.R. spectroscopy: [22]

One part of the sample was mixed with 100 parts of the liquid paraffin, suspended thoroughly and the resulting mixture was placed in sodium chloride cell and scanned in I.R spectrophotometer (Thermo).

Characterization of Aspergillus terreus using blastn

Local soil fungal isolate preliminarily identified in the laboratory was *Aspergillus terreus*. Partial DNA sequencing was done at MTCC Chandigarh and was further characterized and confirmed. B-tubulin was used as

a reference gene for sequencing. The partial gene sequence obtained for B-tubulin from *Aspergillus terreus* was analysed using blastn, followed by partial cds of the data. The query sequence of the local isolate was compared to *Aspergillus terreus* isolate NRRL 255 (beta-tubulin gene.)[23]

Results and Discussion:

In the present study, 79 *Aspergillus terreus* soil fungal isolates named serially from KSV-SUCP-1 to KSV-SUCP-79, were screened for Lovastatin producing potential both by *Neurospora crassa* bioassay. The results of bioassay (Table 1) revealed that *Aspergillus terreus* soil fungal isolate KSV-SUCP-75 has exhibited maximum zone of inhibition which is due to the maximum capacity to produce the drug Lovastatin. The other fungal isolates with decreasing order of their Lovastatin producing potential are KSV-SUCP-24, KSV-SUCP-49, KSV-SUCP-42and KSV-SUCP-2.

Table.1. Yield of lovastatin by *Aspergillus terreus* fungal strains.

S.No	Source of Soil sample	Fungal isolate	Yield of Lovastatin (µg/ml)
1	Garden soil, Mahaboob Nagar (SKC)	KSV-SUCP-2	194
2	Paddy fields, Hyderabad(AUG)	KSV-SUCP-24	289
3	Paddy fields, Warangal(RRM)	KSV-SUCP-49	241
4	Garden Soil, Hyderabad(SMCH)	KSV-SUCP-42	235
5	Pulse fields, Ongole, (ONG)	KSV-SUCP-75	360

The R_f values of the fungal extracts obtained in TLC (using different solvent systems) were also found to be similar to that of the standard drug. (Table.2). TLC confirmation was followed by further estimation of lovastatin at 238 nm. Both sample and the standard exhibited λ_{max} at 238nm. Fig.1.

Table.2. Rf values of standard and test sample in different solvents.

S.NO	Solvent system	Rf. Standard drug	Rf. Sample
1	Dichloromethane Ethyl acetate (70:30)	0.5	0.5
2	Toluene: Ethanol (80:20)	0.701	0.70

3	Ethyl Acetate: Hexane: Acetic Acid (70:30:6)	0.56	0.56
4	Dichloromethane ; Acetic Acid (85:15)	0.6	0.59

A

B

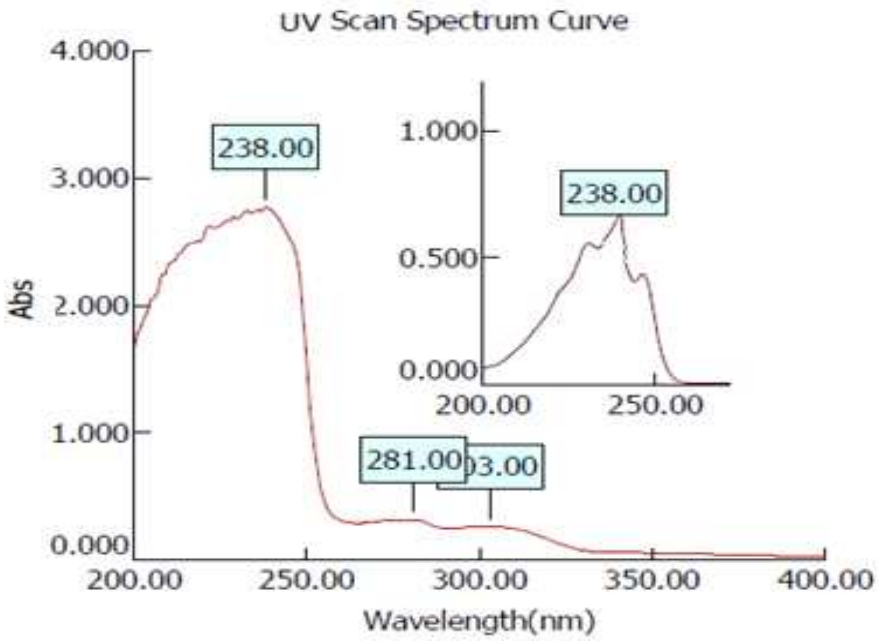

Fig.1. UV spectrum of lovastatin A) Ethyl acetate extract (sample) showing the maximum absorption at 238nm B) Standard lovastatin drug.

HPLC analysis also confirmed the presence of lovastatin in the fungal extract. Retention time of lactone form of standard lovastatin and sample are 5.30 and 5.160 respectively. Fig. 2, and Fig.3. In acid treated broth the drug exists in equilibrium between lactone and hydroxy acid form.[22,] (The retention time corresponding to hydroxy acid form is 1.397). The other peaks in the sample might be due to the presence of impurities or other unidentified compounds of the sample.

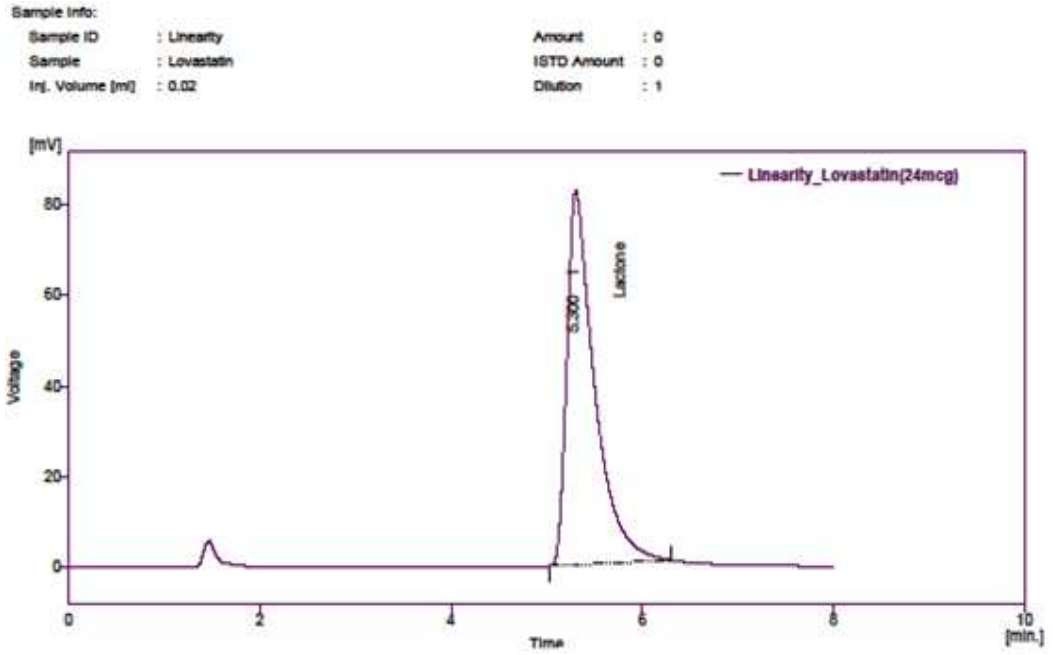

Fig.2 HPLC of Standard Lovastatin drug (Lactone form).

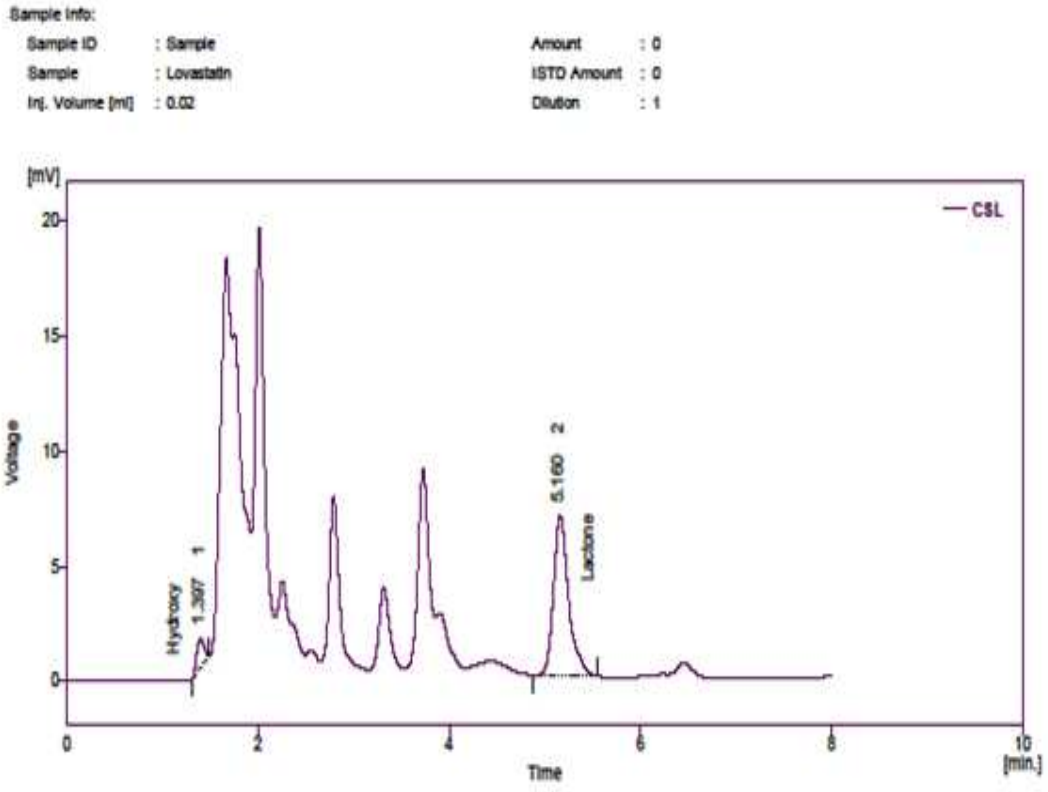

Fig.3 HPLC of sample (Lovastatin in fermentation broth).

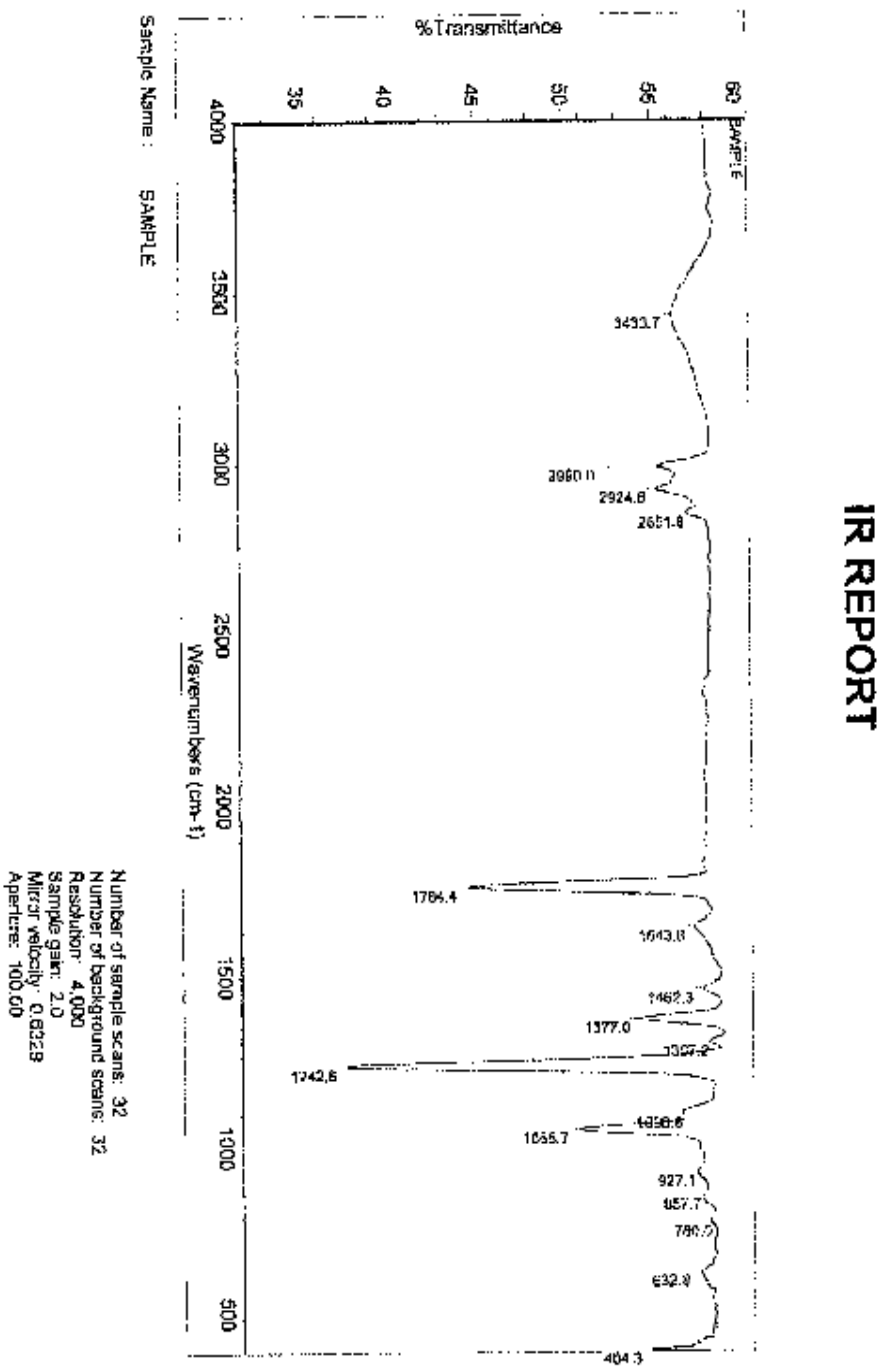

Fig.4 IR spectrum of the sample

Interpretation of I.R spectrum:

Basically lovastatin structure contains the functional groups lactone and hydroxyl groups. The presence of these functional groups is shown in I.R spectrum. Fig.4.

I.R spectrum shows the wave number at 1764.4 which correspond to lactone group of lovastatin and 3433.7 corresponds to that of hydroxyl group.

Results of Genetic analysis: The strain was characterized as *Aspergillus terreus* by MTCC Chandigarh and the assigned accession No.is: MTCC 10831.

Conclusion:

In the present study an attempt was made to isolate potential lovastatin producing *Aspergillus terreus* fungal strains from soil samples. Of the soil fungal isolates, KSV-SUCP-75(MTCC-10831), recorded a maximum yield of 360µg/ml of lovastatin. Results of bioassay and Analytical report (TLC, U.V,HPLC and I.R) confirmed the identity of lovastatin with that of the authentic sample. Gyorgy Szakes et. al reported that the *Aspergillus terreus* fungal strain TUB F-514 produced 140µg/ml in 7 days,[6] N.Jaivel and P. Marimuthu reported JPM3 to have produced 138.4mg/l of lovastatin.[11] Therefore the soil fungal isolate *Aspergillus terreus* (MTCC 10831) is considered as a potential producer of lovastatin and further strain improvement studies and optimization of medium can enhance the production of lovastatin.

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References

1. Lewington S, Whitlock G, Clarke R, et al. Lancet 2007, vol 370, pp1829 –39.
2. Endo A Kuroda, M., and Tsujita.Y , J. Antibiot. (Tokyo), 1976, vol 29, pp 1346-1348.
3. Alberts, AW, Chen J, Kuron G, Hunt V, Huff J, Hoffman C, Rothrock J, Lopez M,Joshua H, Harris E, Patchett A, Monaghan R, Currie S, Stapley E, Alberts SchonbergG, Hensens O, Hirsh field J, Hoogsten K, Liesch J & Springer J. 1980, Sciences of the USA vol 77, pp3957-3961.
4. Alberts AW. Am. J. Cordiol (1988) vol 62:10J-15.

5. Szakacs G.Morovjan G & Tengerdy R.P, Biotechnol. Lett. 1998, vol 20, pp411-415.
6. Manzoni M, Rollini M, Bergomi S & Cavazzoni V. Biotechnol. Lett 1999, vol 21, pp253-257.
7. Manzoni M, Rollini M . Appl. Microbiol Biotechol. 2002, vol 58, pp535-564.
8. Manzoni M, Rollini M, Bergonu S.& Cavazzoni V. Biotechnol Techniques. 1998, vol 12, pp529-532.
9. Lingappa K, Vivek Babu C.S, Siddalingeswar K.G and Pramod T : Ind. J. Microbiol., vol.44 (2): PP 133-135.
10. N.Jaivel and P.Marimuthu. International J. Engineering science and Technol. 2010, Vol. 2 (7) , pp2607-2611
11. Endo A , J. Antibiot. 1979, vol 32, pp852-854.
12. Juzlova, P.,L.Martinkova and V.Kren. J. Ind. Microbiol., 1996, vol.16, pp163-170.
13. Gunde-Cimerman N, Friedrich J, Cimerman A & Benicki. FEMS Microbiol. Lett. 1993, vol. 111, pp203-206.
14. Bilheimers, D.W. Grundy, S.M., Brown, M.S., and Goldstein, J.L. Proc. Natl. Acad. Sci. U.S.A., 1983, vol 80, pp 4124—4128.
15. Novak, N., S. Gerdin and M. Berovic. Biotechnol. Lett. 1997, vol 19, pp947-948
16. Seifert KA: McGraw-Hill, New York; 1990, pp21-51.
17. Raper KB & Fennell D.I. The genus Aspergillus, Williams and Wilkins, New York; 1965, pp.567-577.
18. Kumar MS, Kumar PM, Sarnaik HM & Sadhukhan AK . J. Microbiol Methods 2000, vol 40, pp99-104.
19. Siamak M. Samiee, Nasrin Moazami, Saeid Haghighi, Farzaneh Aziz Mohseni,Saeid Mirdamadi and Mohammad Reza Bakhtiari.Iran. Biomed.J. 2003, vol 7(1), pp29-33.
20. Morovjan G, Szakacs G, Fekete J. Chromatogr. A, 1997, 763:165-172.
21. Bibu Prasad Panda, Saleem Javed and Mohammed Ali.Czech..Food Sci. (2009), Vol 27(5), pp352-360.
22. Watson D. G ;Pharmaceutical Analysis; A text book for pharmacy students and Pharmaceutical chemists; Elsevier Churchill Livingstone; U.K,2005,PP118-119.
23. Louise N, Glass and Gary, Donaldson C, Applied and Environ Microbial, 1995, vol. 61 (4), pp1323-1330.

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