"Isolation and Identification of *Aspergillus terreus* and Production of Lovastatin"

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Abstract

Statins are the secondary metabolites which are produced by some fungal strains and are widely employed for reducing elevated levels of cholesterol in blood plasma Lovastatin is a potent drug was the first statin accepted by United States Food and Drug Administration (USFDA) in 1987 as a hypercholesterolemic drug .Lovastatin, a naturally occurring secondary metabolite is commonly found in foods such as red yeast rice, oyster mushroom, and Pu-erh, although in low concentration, and is primarily used for the cure of dyslipidemia and the prevention of heart-associated diseases as well. Lovastatin is also produced by some specific higher fungi such as *Aspergillus terreus, Pleorotus ostreatus,* and closely associated *Pleorotus* species. Production of lovaststin was done by using solid state fermentation by using wheat bran and rice bran .Maximum yield was obtained when wheat bran was used as a substrate.

Key words : Lovastatin,*Aspergillus terreus*,wheat bran ,rice bran.

INTRODUCTION

Hypercholesterolemia is a major contributor to atherosclerosis and its clinical sequelae, myocardial infarction, ischemic stroke, and peripheral vascular diseases. Hypercholesterolemia is the presence of high levels of cholesterol in the blood. Cholesterol is a waxy fat-like substance and is a major class of lipid, so it gets into the blood by lipoproteins.A high level of lipoproteins is unhealthy. A high level can result in an elevated risk of atherosclerosis and coronary heart disease. The high levels of lipoproteins are often influenced by a combination of genetic and environmental factors such as obesity or dieting habits. During the past decades, cholesterol control by a variety of drugs has yielded a significant reduction in cardiovascular mortality. Since more two third of total body cholesterol synthesized *de novo,* inhibition of biosynthesis is considered as an important way to control its level in blood. Statins,a class of fungal

secondary metabolites, competitively inhibit 3-hydroxyl-3-methylglutaryl coenzyme-A reductase (HMG-CoA reductase), the rate limiting step in cholesterolbiosynthesis, significantly lowering the blood cholesterol level in human and animals, and thusbecoming the compound of choice for the purpose. (R. H. Patil.et.al.2011)

Lovastatin has been a potent drug for lowering the blood cholesterol and it was the first statin accepted by United States Food and Drug Administration (USFDA) in 1987 as a hypocholesterolemia drug. It is a competitive inhibitor of HMG-CoA reductase, which is key enzyme in the cholesterol production pathway.

Lovastatin, sold under the brand name Mevacor. Lovastatin was patented in 1979and approved for medical use in 1987.

It is on the World Health Organization's List of Essential Medicines. It is available as a generic medicines. In 2019, it was the 95th most commonly prescribed medication in the United States,with more than 8 million prescription.

Among the most commonly prescribed drugs to lower cholesterol levels are statins. Statins is a group of drugs that specifically inhibits 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, a rate-limiting enzyme involved in cholesterol biosynthesis in the liver. Thiseffectively lowers the plasma low-density lipoprotein cholesterol (LDL, bad cholesterol), while increasing high-density lipoprotein (HDL, the good cholesterol) and inhibits the subsequent increase in hepatic LDL receptor expression. Subsequently, it can reduce atheroma accumulation and swelling of arterial wall thrombus and atherosclerosis formation, improves endothelial function and suppressing inflammatory reaction. Recently, preliminary studies also claimed that statins can potentially treat other conditions, such as osteoporosis, Alzheimer's, and cancer.

Lovastatin is a natural statin, largely produced by fungi through cultivation process. Statin wasfirst isolated from fungal family, including the *Aspergillus, Monascus* and *Penicillium* family. The first natural statin, compactin, was isolated in 1976 when a metabolite produced by *Penicillium citrinum* was found to inhibit the activity of cholesterol precursor in rat liver extract.(Muhamad Hafiz Abd Rahim et.al.2015)

Materials and Methods

Identification and isolation of *Aspergillus terreus*

Sample collection:

Total 15 soil samples were collected from the various locations. The soilsamples were collected up to the depth of 1-5 cm from the soil surface.

Cultivation:

Fungal stock solution were prepared by diluting 1 gm of soil in 9 ml of of sterile distilled water and shaken by using vortex. From the stock solution, 1 ml was used to prepare final volume of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} by serial dilution method. This dilutions were spreadinoculated on PDA and this plates were incubated at room temperature for 3 to 5 days, after that the results were recorded.

Plates were observed and visual identification and staining was performed using lactophenol cotton blue stain. The isolated colonies were selected and inoculated them on fresh sterile PDA plates followed by incubation. Then the pure culture was maintained on potato dextrose agar slant.

Harvesting of Spores

After incubation for 48 hrs, the growth from slant was aseptically scrapped and harvested in 50 ml sterile (saline +1% SLS) solution.

Production of lovastatin by solid state fermentation:

Fermentation medium:

Production was carried out using different solid agro based residues rice bran and wheat bran to evaluate their support for lovastatin production. Sterile good quality low density residues were taken. Containing 25 gram of rice bran and wheat bran separately in500ml of conical flasks containing 100 ml of distilled water in each flask. Spores of *Aspergillus terreus* were inoculated in both flasks under aseptic conditions. Incubated at room temperaturefor 10 days.

Inoculation of spore suspension

1 ml of 10^6 spores/ml of spore suspension taken from best sporulating medium wasused for inoculation of rice bran and wheat bran medium.

Recovery:

Extraction of lovastatin from solid state fermentation:

For wheat bran:

25 ml of butanol in 25 ml of distilled water (1:1,v/v) was added to the fermentation flask and kept it on rotary shaker at 200 rpm at 30^0 C for 2 hours. After it the mixture was centrifuged at 5000 rpm for 20 min in a centrifuge and then the supernatant filterthrough Whatman filter paper no. 1. Then the extract was dried to form a powder.

For rice bran:

50 ml of butanol was added to the fermentation flask and kept it on rotary shaker at 280 rpm at 30^0 C for 2 hours. After it the mixture was centrifuged at 5000 rpm for 20 min ina centrifuge and then the supernatant filter through Whatman filter paper no. 1. Then the extractwas dried to form a powder.

Analysis of Lovastatin using UV Spectrophotometry:

Preparation of standard lovastatin stock solution:

The standard lovastatin powder was brought from the market.

For the preparation of stock solution 1 mg of lovastatin powder was dissolved in 1 ml of acetonitrile solution i.e. 1mg/ml stock solution. it was further diluted to 2, 4, 6,……10µg/ml. Reading of all the concentration were taken at 232 nm wavelength..

This proportion were used for all the UV spectrophotometric analysis.A standard graph was plotted using standard lovastatin.

Analysis of pure lovastatin:

Working standards of lovastatin were prepared and the absorbance was read at 232 nm using a UV-visible spectrophotometer. The zero-order absorption spectra were obtained over the wavelength range of 200-600 nm using the appropriate dispersion medium in a quartzcuvette (1 cm path length) at 1.5 nm slit width (Δλ).

Analysis of lovastatin:

The extracted powder obtained from the recovery process of both rice bran and wheat bran were dissolved in acetonitrile solution. The 1 mg powder of extract werediluted in 1 ml of acetonitrile. The absorbance were taken at 232 nm because it suggest better identification of lovastatin from other product. The blank were adjusted by acetonitrile solution. Crude lovastatin sample in the acetonitrile was checked for absorbance at 232 nm wavelength. Thus, concentration of crude product obtained from rice bran and wheat bran wasdetermined with the help of standard graph.

Detection of Lovastatin produced by *Aspergillus terreus* using Paper chromatography:

Crude lovastatin obtained was spot applied on Whatman filter paper No.1. The

paper was kept in chromatographic chamber containing equilibrated solvent system. The solvent for paper chromatography was acetonitrile: water (9:1 ratio). The paper was then driedand observed for yellow spot under U.V

light.

Determination of Rf value:

The distance travelled by a solute Rf =

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The distance travelled by a solvent

Results And Discussions

1) Isolation and Identification

Isolation of *Aspergillus terreus* was carried out on PDA.

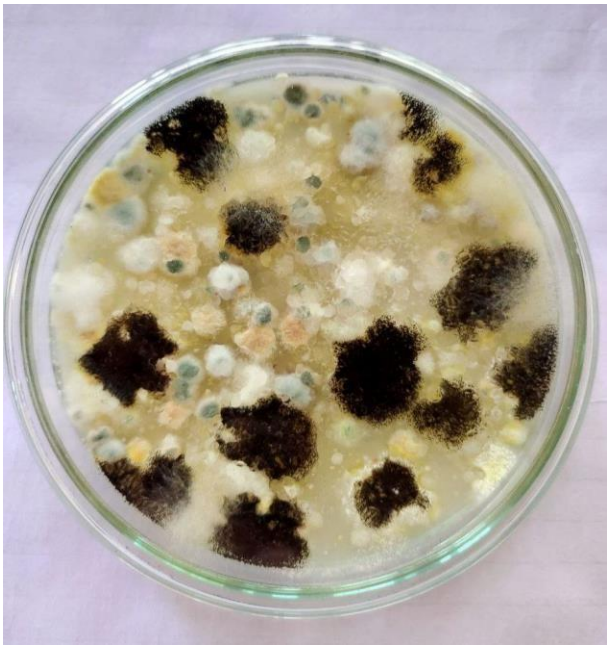

Fig. No.5.1 Isolation of *Aspergillus spp.*

Fig. No.5.2 Isolation of *Aspergillus terreus*

Table no. 5.1 Results of the colony characterizations

Isolate	Size	Shape	Colour	Margin	Elevation	Opacity	Spore Colour
a.	3-4 mm	Globose	Sand Brown	Filiform	Umbonate	Opaque	Sand Brown
b.	4-5 mm	Globose	Sand Brown	Filiform	Umbonate	Opaque	Sand Brown
c.	3.5-4.7mm	Globose	Sand Brown	Filiform	Umbonate	Opaque	Sand Brown

2) Microscopic Observation:

By performing the cultural, morphological and microscopic observation the isolate wastentatively identified as *Aspergillus terreus*.

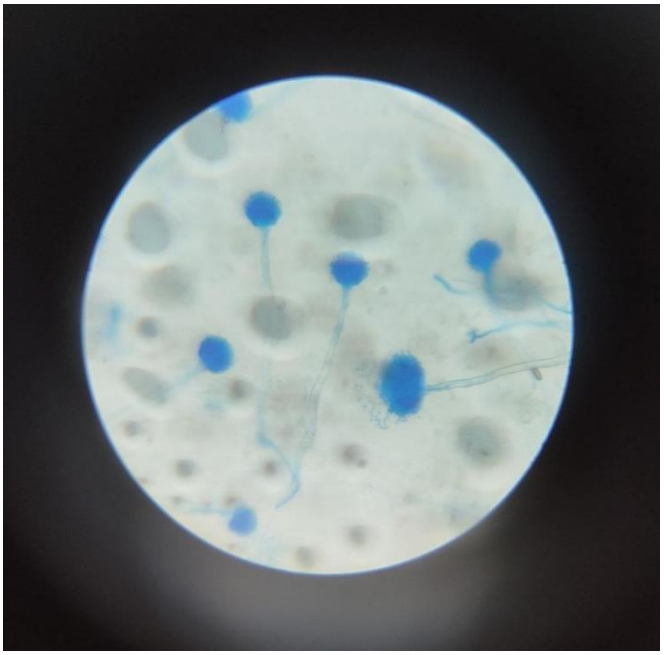

Fig. No. 5.3 Result of the microscopic observation

3) Solid State Fermentation and Detection of Lovastatin:

Solid state fermentation of Lovastatin was carried out using two media viz. rice bran and wheat bran. After 10 days of incubation of both these media, Lovastatin was extracted and purified by appropriate technique. It was dissolved in acetonitrile and further detected by various methods which are discussed below

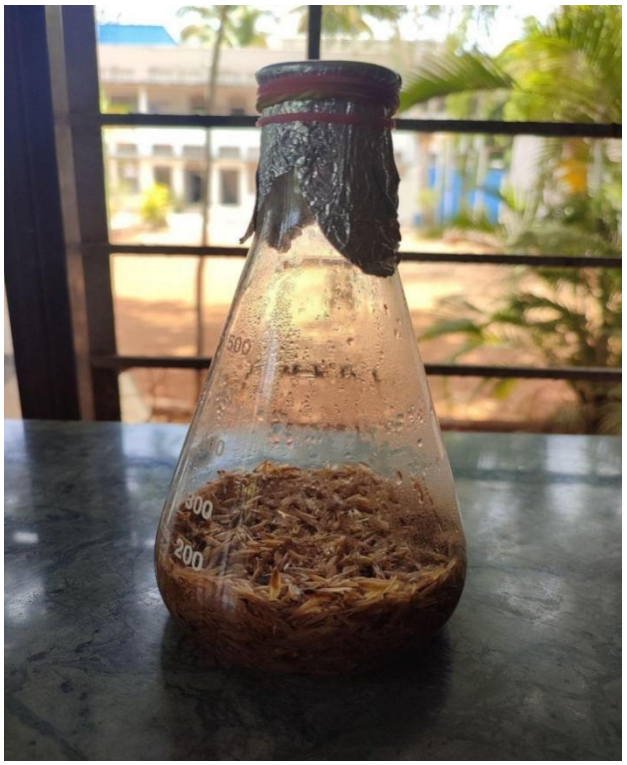

Fig No.5.4 Fermentation media of lovastatin by using rice bran

Fig No. 5.5 Fermentation media of lovastatin by using wheat bran

4) Detection of Lovastatin by using Paper chromatography

A. Detection of lovastatin by paper chromatography:

On observing chromatography under U. V. light, two yellow coloured spots were observed. By comparing Rf values of both the spots it becomes clear that, crude solution in acetonitrile contains Lovastatin. Rf values of crude lovastatin and that of standard lovastatin are given in table

Table no. 5.2 Results of paper chromatography are as shown below:

Sr. No.	Lovastatin extracted from	Rf value
1.	Rice bran	0.56
2.	Wheat bran	0.62
3.	Standard (control)	0.57

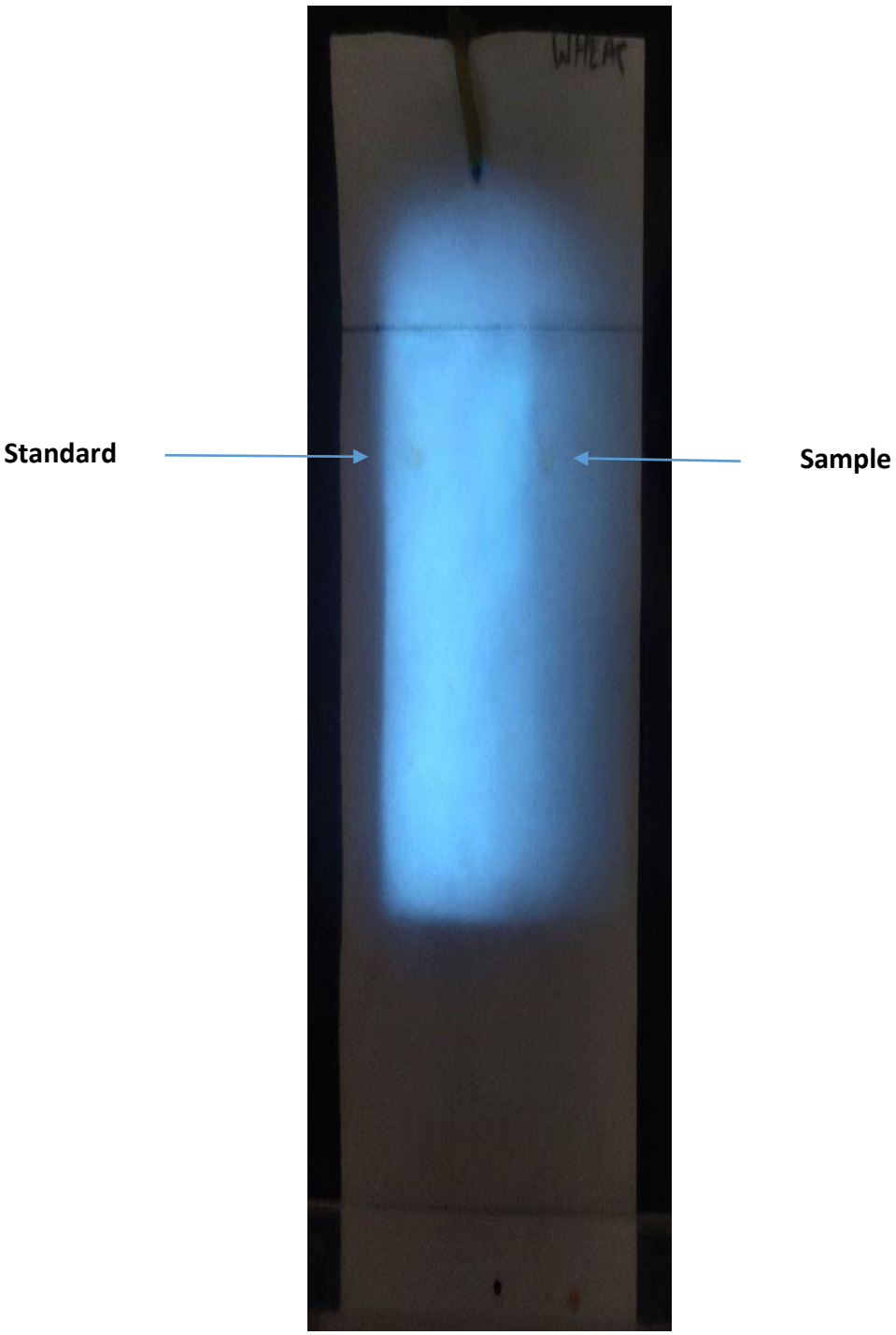

Fig No. 5.6 Result of chromatography of lovastatin extracted from wheat bran

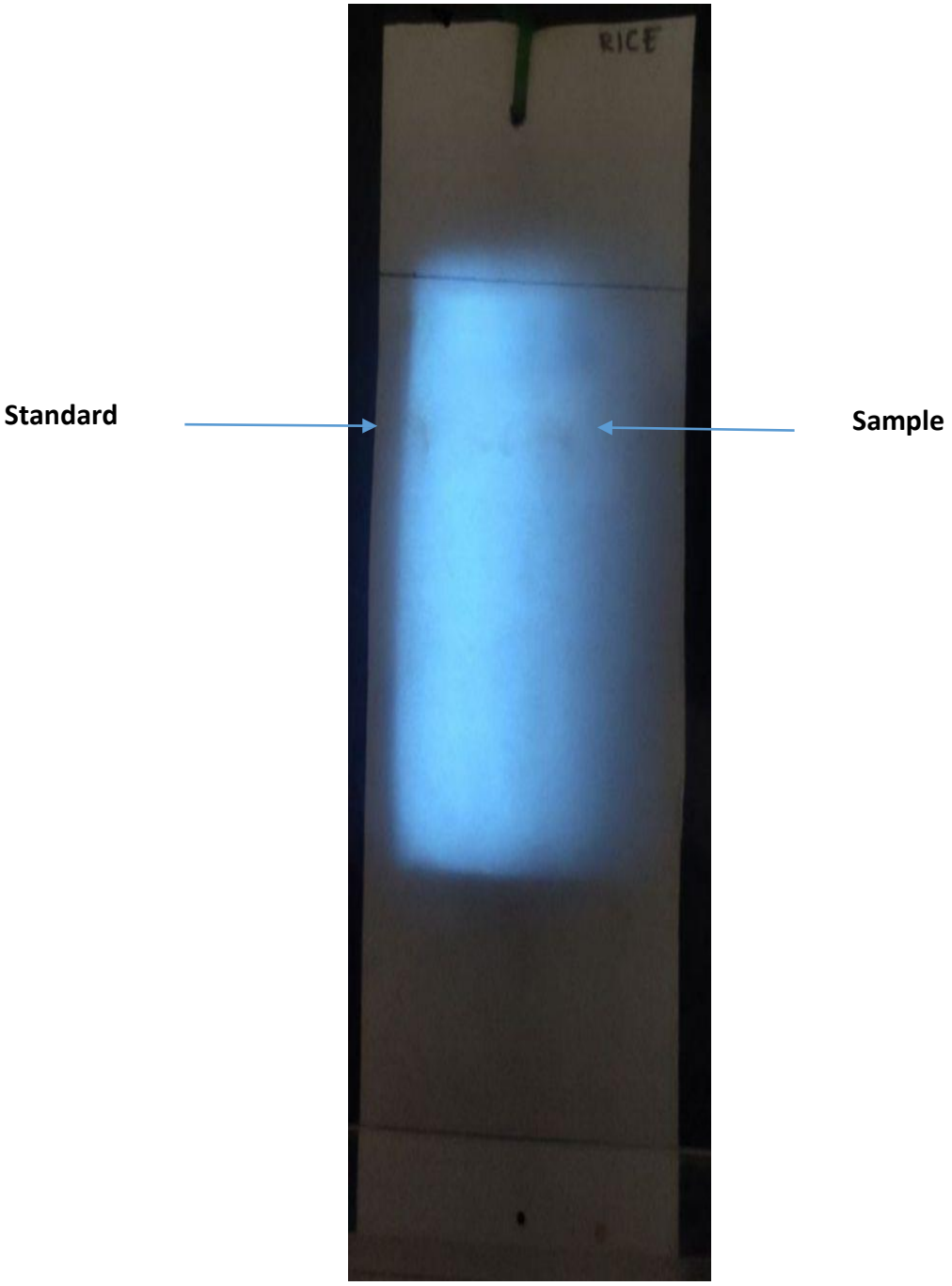

Fig No. 5.7 Result of chromatography of lovastatin extracted from rice bran

5) Measurement of Lovastatin produced by U. V. SpectrophotometricAnalysis :

Values of absorbance of different standard concentrations of Lovastatin at 232 nm. are given in table no.5.3 and also plotted in fig. 5.1. Values of OD of crude Lovastatin obtained from two different substrates viz. Rice bran and wheat bran are shown in tableno. 5.4

By plotting these values on standard graph, concentration of crude lovastatin wasdetermined which is shown in table no. 5.5

6) UV spectrophotometric analysis of market available lovastatin as standard areshown below:

Table no. 5.3 Results of the standard lovastatin

Sr. No.	Concentration of standard (ug/ml)	Absorbance at 232 nm
1.	0	0
2.	2	0.185
3.	4	0.345
4.	6	0.526
5.	8	0.722
6.	10	0.907

7) Results of UV spectrophotometric analysis of lovastatin production produced by *Aspergillus terreus* from Rice bran and wheat bran are shown below.

Table no. 5.4 Results of spectrophotometric analysis of lovastatin by using rice and wheat bran

Sr no.	Sample	Absorbance at 232 nm
1.	Rice bran	0.297
2.	Wheat bran	0.598

According to the spectrophotometric analysis of lovastatin wheat bran shows the higher absorbance as compared to rice bran.

8) Yield of lovastatin from standard graph

Table no.5.5 result of lovastatin by using rice and wheat bran

Sr. no.	Substrate used for lovastatin production	Yield of lovastatin
1.	Rice bran	0.34 mg/ml
2.	Wheat bran	0.67 mg/ml

According to the spectrophotometric analysis of standard lovastatin wheat bran shows the higher yield as compared to rice bran.

Spectrophotometric analysis of lovastatin

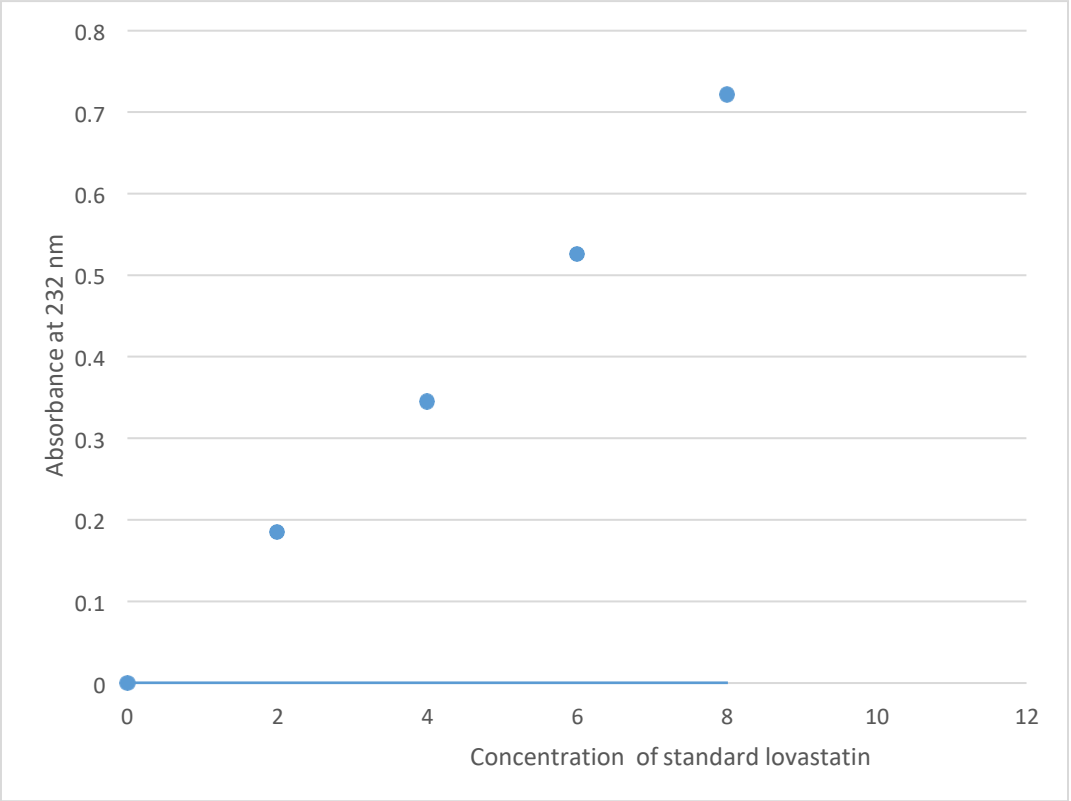

Fig no. 5.8 Standard graph of lovastatin production

Summary and Conclusion:

Summary

The isolated strain of fungal culture of *Aspergillus terreus* was used to carry out the project work. Fungal culture prepared on PDA slants was first harvested and measured for spore count by hemocytometer.Solid State Fermentation (SSF) technology was applied for Lovastatin production . During this course,two crude substrate viz. Rice bran and wheat bran were used as production medium as well as to their productivity. Lovastatin was extracted and purified. This crude form of drug was detected by performing paper chromatography. Concentration of lovastatin was determined using U.V. spectrophotometeric analysis. Thus the possibility of use of crude media for lovastatin production was checked.

Conclusion

Following conclusion could be drawn from the work:

1. Rice bran as well as wheat bran be used for lovastatin production using Solid state fermentation (SSF) technology.
2. Rice bran gives 0.34 mg/ml of yield of lovastatin whereas, wheat bran gives 0.67mg/ml. Thus wheat bran is the better production medium as compared to Rice bran
3. Crude powder purified from production medium proved to contain lovastatin by chromatography, U.V spectrophotometric method.

References

- A. seenivasan, s. subhagar, r. aravindan* and t. viruthagiri Microbial Production and Biomedical Applications of Lovastatin Biochemical Engineering Laboratory, Department of Chemical Engineering, Annamalai University, Annamalai Nagar-608 002, India
- Donald F. Gerson, Xinfa Xiao, "Process for the production of lovastatin using Coniothyrium fuckelii." U.S. Patent US5409820, issued January, 1984.Donald F. Gerson, Xinfa Xiao, "Process for the production of lovastatin using *Coniothyrium fuckelii*." U.S. Patent US5409820, issued January, 1984.
- Hassan hajjaj,* peter niederberger, and philippe duboc Nestle´ Lovastatin Biosynthesis by *Aspergillus terreus* in a Chemically Defined Medium Research Center, Nestec Ltd., Vers-chez-les-Blanc, CH-1000 Lausanne 26,Switzerland
- Hedayati, M.T.; Pasqualotto, Warn; Bowyer, Denning (2007) "*Aspergillus flavus*: human pathogen, allergen and mycotoxin producer". Journal of Medical Microbiology. 153 (6): 1677–1692. doi:10.1099/mic.0.2007/007641-0. PMID 17526826. Retrieved 9 November 2013.
- M. Bizukojc Æ S. Ledakowicz The morphological and physiological evolution of *Aspergillus terreus* mycelium in the submerged culture and its relation to the formation of secondary metabolites Received: 1 December 2008 / Accepted: 28 July 2009 / Published online: 9 August 2009 Ó Springer Science+Business Media B.V. 2009

- PanelH.R.ValeraaJ.GomesaS.LakshmibR.GururajabS.SuryanarayanbD.Kumar, Lovastatin production by solid state fermentation using *Aspergillus flavipes* Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology, New Delhi 110016, IndiabBiocon India Ltd., Hosur Road, Bangalore, Karnataka, IndiacSunPharma Advanced Research Centre, Vadodara, Gujarat, India
- Ravindra H. Patila, Prakash Krishnanb and Vijay L. Maheshwarib Production of Lovastatin by Wild Strains of *Aspergillus terreus*,*a Department of Microbiology and Biotechnology, R. C .Patel Arts, Commerce and Science College, Shirpur 425405 (MS), India b Department of Biochemistry, School of Life sciences, North Maharashatra University,Jalgaon 425001 (MS), India
- Rashmi Dikshit & Padmavathi Tallapragada Bio-synthesis and screening of nutrients for lovastatin by *Monascus sp.* under solid state fermentation Revised: 25 November 2014/Accepted: 8 December 2014 /Published online: 11 February 2015 # Association of FoodScientists & Technologists (India) 2014
- Shi-Weng Li, Hong-Ping Song & Yan Leng To cite this article: Shi-Weng Li, Hong- Ping Song & Yan Leng (2014) Rapid determination of lovastatin in the fermentation broth of *Aspergillus terreus* using dual-wavelength UV spectrophotometry Rapid determination of lovastatin in the fermentation broth of *Aspergillus terreus* using dual- wavelength UV spectrophotometry, Pharmaceutical Biology, 52:1, 129-135, DOI: 10.3109/13880209.2013.833947
- Sumel Ashique, Dr. Sachin Tyagi, Raneet Das, Ayan Goswami, Jayita Roy, Mr. Mir Samim Ali, Mr. Alok Semwal Shashwat Publication, 05-Jan-2022 - Science - 192 pages