Original Article

Optimization Of Carbon Sources For Maximal Production Of Lovastatin By Forest Soil Fungal Isolates Under Liquid State Fermentation (LSF)

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Abstract

Most fungal secondary metabolites are remarkably complex bioactive compounds. Soil Fungi produce a large variety of compounds mainly through the polyketide biosynthesis pathway. 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. The main objective of the study is screening of various fungal species and optimization of different carbohydrate sources for maximizing lovastatin production by *Aspergillus spp* using Liquid State Fermentation (LSF). The soil samples were collected on the random basis from different locations around Pachmarhi, Madhya Pradesh (India). The pure cultures of these fungal isolates were screened for production of statin or lovastatinwere evaluated by yeast inhibition test. Potential species were subjected to *in vitro* carbohydratesoptimization in LSF fermentation and concentration of Lovastatin was estimated by HPLC. The pure culture of indigenous fungal isolated were screened for their capability of producing statin orlovastatin like substances was evaluated by adopting yeast inhibition model. For this ATCC cultureof *Candida albicans* and *Saccharomyces cerevisiae* were used as model organism against which statin screening fungal isolates that showed antagonistic activity. Fungal isolate were subjected to *in vitro* production of lovastatin under the influence of different carbohydrate sources at a concentration of 30gm per litre in LSF medium. The quantity of lovastatin produced by *Aspergillus spp* using HPLC was found to be 1.48, 0.94, 8.19, 1.69, 0.07, percentage using Sucrose, Dextrose, Mannitol, Lactose and Maltose as source of carbohydrate respectively. Production of lovastatin was maximum using mannitol as a source of carbohydrate.

Keywords: Soil fungi, Statin, Lovastatin, HPLC, LSF

INTRODUCTION

In the development of medical science, microorganism have a major impact since the discovery as they not only cause infections but also produce certain organic compounds that cure infections and help in treatment of variety of non-infectious diseases (Gunathilake, 2017; Singh, *et al*., 2017). Though, microbes are ubiquitous, but their metabolic capabilities are greatly influence by the habitatthey survive with unique conditions of pH, temperature, pressure, oxygen, light, nutrients and salinity, there is a high potential for those to produce metabolites exhibit special biological activities (Gunathilake, 2017). Microorganisms are of immense importance to environment and essential to all life forms, and are primary source of nutrients and act as chief recycler in environment (Bisen *et al.*, 2012). Forthe sake of ever increasing world population microbes have been known extensively for their potential in the development of bioprocess technologies for production of secondary metabolites which are organic compounds that form at the end or near the stationary phase of growth, and are not directly associated with growth, development, and reproduction of microorganisms itself. These products include nutrition supplements such as, vitamins and amino acids, organic acids, agriculturally important metabolites, enzymes, flavoring agents, coloring agents and pharmaceutical and healthcare products like antimicrobial agents, antiparasitic agents, antitumor, enzyme inhibitors and immunosuppressive etc. (Demain 1999; Demain 2007). Soil is the reservoir of the quantity of microorganism where microbial load in soil is dependent upon intricate network between of physical and biological factors. Numerous microorganisms (Bacteria/Fungi/Actinomycetes) exhibits diversity survives in soil to produce enthralling and structurally complex bioactive products of pharmaceutical importance.

MICROBIAL METABOLITES

Microorganisms are present in extremely large sphere of environment and thrive from abyssal zone to stratosphere (at heights up to 60 km) and in a wide range of temperatures ranging from arctic ice to boiling volcanoes (Imshenetsky *et al*., 1978; Wainwright *et al*., 2006). These microscopicorganisms are used in the preparation of variety of foods and also used as a source of food and feed supplements. For example, amino acids are obtained from C*orynebacterium, Brevibacterium* and *Escherichia coli*; vitamins from *Propionibacterium* and *Pseudomonas*; organic acids from *Aspergillus, Lactobacillus, Rhizopus* and enzymes from *Aspergillus, Bacillus* (Shimizu 2001; Gurung *et al*., 2013;

Mahmood 2015; Sun *et al*., 2015). The treatment of hypercholesterolemia is targeted by decreasing the low density lipoprotein by medications. A wide variety of biological active compounds are produced by fungi (De Silva *et al*. 2012a, 2012b, 2013) including statins (anti-cholesterol compounds). Statins are the inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, have revolutionized the treatment of hypercholesterolemia. They are the most efficient agents for reducing plasma cholesterol, being also appreciated for their good tolerance. Angiographic studies have demonstrated that these compounds reduce the progression and may induce the regression of atherosclerosis. These effects were translated in significant cardiovascular morbidity and mortality reductions in many clinical trials (WOSCOPS, AFCAPS/TexCAPS, HS, CARE, LIPID, and HPS) (Vaughan, *et al*., 2000). The beneficial effects of the HMG-CoA reductase inhibitors are usually attributed to their capacity to reduce the endogenous cholesterol synthesis, by completely inhibiting the principal enzyme involved (Hunninghake, 1992). Since mevalonate, the product of HMG CoA reductase reaction, is the precursor not only for cholesterol, but also for many other non-steroidal isoprenoidic compounds, inhibition of this key enzyme may result in pleiotropic effects. They have been divided into two categories, involving: directly lipids, or intracellular signaling pathways. The first category includes: inhibition of cholesterol biosynthesis, increased uptake and degradation of low density lipoproteins (LDL), inhibition of the secretion of lipoproteins, inhibition of LDL oxidation, and inhibition of the scavenger receptors expression (Bellosta, *et al*., 2000). Statins modulate a series of processes leading to reduction of the accumulation of esterified cholesterol into macrophages, increase of endothelial NO synthetase, reduction of the inflammatory process, increased stability of the atherosclerotic plaques, restoration of platelets activity and of the coagulation process (Bellosta, *et al.,* 2000).

MATERIALS AND METHODS

Sample collection

Location: Pachmarhi Bioreserve (between latitude 22˚11’ to 22˚56’ N and 77˚47’ to 78˚52’ E longitude). The soil samples for study were collected on random basis from different locations in and around Pachmarhi. The details of sampling location are mentioned in Table 1.

Table 1: The different points of soil sampling from in and around Pachmarhi Bioreserve

S.N.	Sampling Points	Sample Description	Sample Code
1.	Bade Mahadev	Black soil, granulated form	A1
2.	Handi Khoh	Black soil, granulated form	A2
3.	Gupt Mahadev	Black soil, granulated form	A3
4.	Baariaam Talab	Black soil, powder form	A4
5.	Pachmarhi Biosphere 17 Km fromTown	Black soil, granulated form	A5
6.	Matkuli	Black soil, powder form	A6
7.	Core Jungle Area	Black soil, granulated form	A7

Isolation & pure culture preparation

For isolation and screening of fungi from collected soil samples, potato dextrose agar media (M096 HiMedia) and sabouraud dextrose agar media (M063 HiMedia) were used, Table 2.

Table 2: Composition of potato dextrose agar media (M096 HiMedia)

S.N.	Ingredients	Quantity in Grams/Litre
1.	Potatoes, infusion from	200 gm
2.	Dextrose	20 gm
3.	Agar	15 gm
4.	Final Ph	5.6±0.2

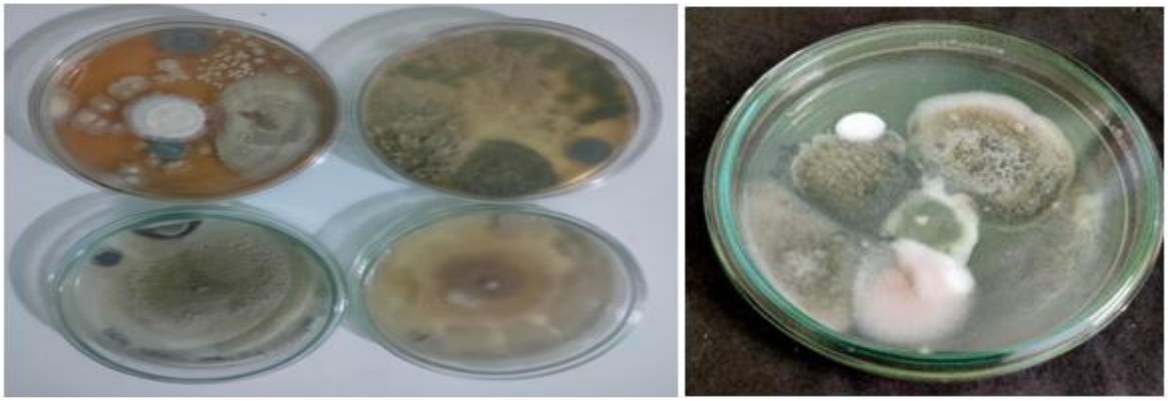


Image 1: Isolates from soil samples

The isolation of soil fungi was carried out by routine microbiological techniques maintaining all aseptic procedures. Each soil samples were serially diluted and a dilution of 10^7 and 10^9 were used to inoculate the culture media by spread plate technique. The culture plates were incubated for 3to 5 days at 27˚C in a BOD incubator. The 20 primary culture plates were established, out of whichthe most repeated fungal colonies were picked up for further studies.
The pure cultures of these fungal isolates were prepared by repeated sub-culturing on fresh plates of Potato Dextrose Agar (PDA) media on similar culture conditions as earlier.

Identification of the most efficient lovastatin producer isolates

The pure cultures of indigenous fungal isolates were screened for their capability of producing statin or lovastatin-like substances were evaluated by adapting the yeast inhibition model. For this ATCC culture of *Sacchromyces cereviceae* was used as a model organism against which statin-secreting fungal isolates would show antagonistic activity. *Aspergillus spp* isolates were separately cultured in SD broth to the prepared sporeless culture at similar incubation conditions for 1 week. The crude fermentation extract of fungal isolate were prepared by centrifugation at 5000 rpm for 20 minutes at ambient temperature. The yeast culture broth was used to inoculate the PDA media culture plates with the help of sterile cotton swab. The crude fermentation extract of fungal isolate were inoculated at the rate of 100 µl as patch. The culture plates were then incubated for 72 hours at 27˚C in a BOD incubator. The concentration of standard Lovastatin with respect to the zone of inhibition against indicator organism were observed and used to calculate the presence of Lovastatin in the crude sample.

Image 2: Patch screening for potential lovastatin producing fungal isolate

Extraction of the lovastatin for bioassay

Pure culture of *Aspergillus* isolates showed yeast inhibition activity. A pure culture is now used for *in-vitro* production of Lovastatin in liquid state fermentation (LSF). Bioassay(disc diffusion method) due to fermentation extract as inhibition of yeast cells due to lovastatin produced by selected indigenous fungal isolates is observed for confirmation.

HPLC instrument and reagents

The quantitative estimation of statin or say more specifically lovastatin produced by selected test fungal isolates in liquid state fermentation at various parameters of optimization was done by HPLC technique.

Reagents and chemicals

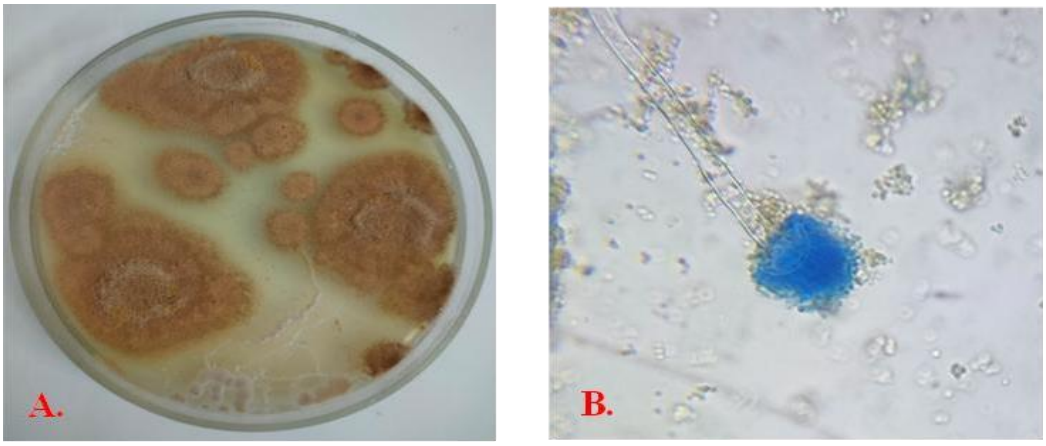
Standard Lovastatin was procured from HiMedia Pvt Ltd. Phosphoric acid, methanol, acetonitrile and water were of HPLC grade and purchased from Merck Ltd, New Delhi, India. Water used was of HPLC grade water from Merck Ltd, New Delhi, India.

Instrumentation

A double beam UV-Vis Spectrophotometer model of Labindia 3000+ with 1cm matched quartz cells was used for determination of λmax of standard marker compound “Lovastatin” which was utilized for estimation of marker compound in given sample. The Shimadzu LC10 HPLC system (Shimadzu Ltd, Japan) consisted of a pump (Shimadzu LC10AT) a U.V. Visibledetector (Shimadzu SPD10A), a reverse phase C18 Targetsil column with dimensions 250×4.6 mm, & particle size 5µm (Wesley Technologies, USA) and N2000 software for chromatography data analysis (Science Technology, Hangzhou, China).

RESULTS

Morphological characteristic of *Aspergillus spp*



Isolate C2 on Culture Plate **Isolate C2 Microscopy**

Image 3: Identification of this fungus was done according to classification of Domsch *et.al.* 1980. Figure shows

Colony morphology and microscopic images of *Aspergillus spp*.

Colony on PDA media is cinnamon coloured, raised looks velvety to granular and filamentous though cottony white areal hyphae also appear in older colonies. The margins are irregular to pinpoint type. The culture grows with moderately in size of 2.5 to 4 cm in diameters within 7 to10 days. Mycelia are slender septate, and branching is also there, with upright conidiophore, Conidial head is compact, which form columns later with biseriate arrangement of phialides while spores are globose to subglobose. Based on the colony morphology and microscopic characteristics the fungal isolates may be *Aspergillus terreus, Aspergillus austwickii, or Aspergillus aflatoxiformans.*

Bio assay of optimized fermentation extract (LSF)

The bioassay by inhibition activity was performed with the fermentation extract of tested fungal isolate against the two yeast species namely *C. albicans* (MTCC-227) and *S. cerevisiae* (MTCC- 170) by disc diffusion method according to Dikshit, and Tallapragada, (2015); Pandey, et al., (2018). Bioassay (disc diffusion method) due to fermentation extract as inhibition of yeast cells due to lovastatin produced by selected indigenous fungal isolates is observed for confirmation. The Zone of inhibition due to tested fungal extract was observed in C. albicans (MTCC-227) was13mm diameter and in S.cereviceae (MTCC-170) was 14 mm in diameter compare with zone of inhibition by standard lovastatin which comes to be 17 mm and 18 mm against C. albicans and S.cereviceae when the filter paper disc was impregnated with standard lovastatin 10 µl solution of 100 µg/ml concentration Table 3

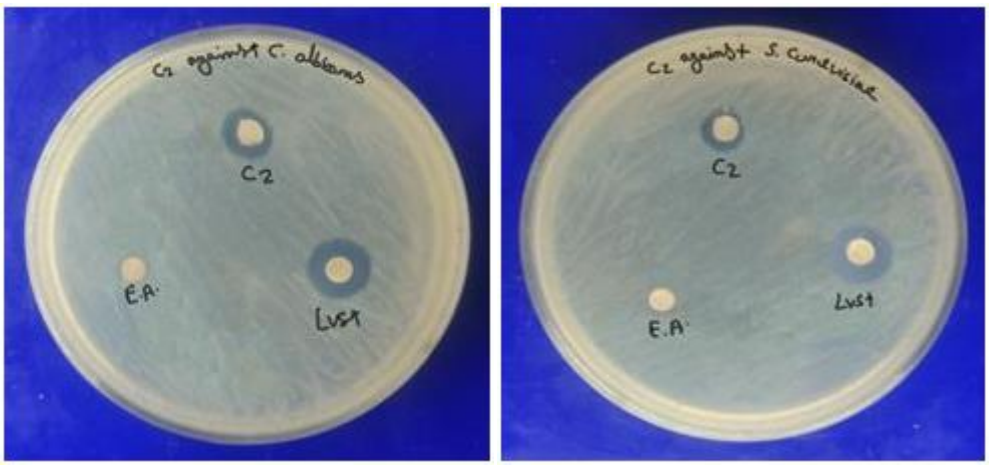


Image 4 : The bioassay by inhibition activity with the fermentation extract of testedfungal isolate against the two yeast species namely C. albicans (MTCC-227) and S. cerevisiae (MTCC-170) by disc diffusion

Table 3: Zone of inhibition (Φ in mm) against test Yeast strain by disc diffusion

S.N.	Isolated Code	Zone of inhibition (Φ in mm) against test Yeast strain by discdiffusion	
		***C. albicans* (MTCC-227)**	***S.cereviceae* (MTCC-170)**
1	C2	13 mm	14 mm
2	Lvst	17 mm	18 mm

Quantitative estimation of lovastatin by HPLC

The percentage concentration of lovastatin produced by *Aspergillus* using different carbon sources such as Sucrose, Dextrose, Mannitol, Lactose, and Maltose respectively were estimated through HPLC. Production of Lovastatin was maximum using Mannitol as a source of Carbohydrates. Image 5 shows the chromatogram of the lovastatin standard used in HPLC.

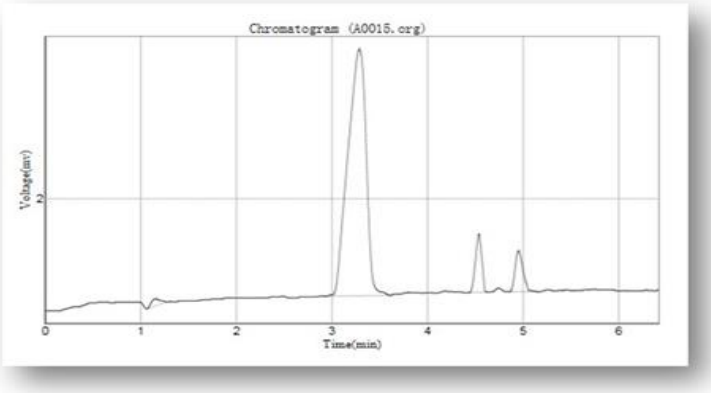


Image 5: Chromatogram of standard Lovastatin marker generated on ShimadzuHPLC system LC10, LC10AT with retention time at 3.29±0.5 min

Table 4 : Percentage lovastatin production due to use of different carbon sources ata concentration of 30gm per litre in LSF medium estimated on Shimadzu HPLC system LC10, LC10AT with retention time at 3.29±0.5 min

S.M.	Fungal Isolate	Percentage Lovastatin Production due to use of different carbonsources at a concentration of 30gm per litre in LSF medium				
		Sucrose	Dextrose	Mannitol	Lactose	Maltose
1	*Aspergillus spp*	1.48	0.94	8.19	1.69	0.07

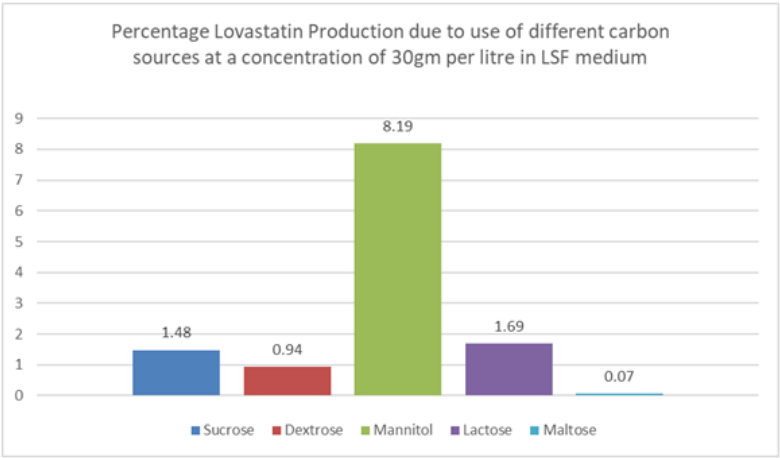


Image 6 : Comparative graph of percentage lovastatin production due to use of differentcarbon sources at a concentration of 30gm per liter in LSF medium estimated on Shimadzu HPLC system LC10, LC10AT with retention time at 3.29±0.5 min

DISCUSSION

In recent years, researchers have focused much on SSF for commercial production of industrially important metabolites. Wheat bran is generally considered as a complete solid substrate for growth and metabolite production of microbes consisting of protein, fats and polysaccharides such as arabinoxylans, cellulose and lignin, but, however, lacks readily soluble sugars (Stevenson *et al*., 2012). The aim of the present work was to study the feasibility of Liquid state fermentation (LSF) as a promising technique influence of few growth supplements such as readily soluble sugars as cheaply available fermentation media for the growth of lovastatin producing isolates. In the present study, of 05 carbon sources tested, such as Sucrose, Dextrose, Mannitol, Lactose, Maltose. Highest yield of Lovastatin *i.e*. 18.15% is in Mannitol as a Carbon source. It is obvious that mannitol being a readily soluble monosaccharide gets metabolized by the fungal isolates rather quickly although interestingly, On the contrary, none of the disaccharides except Lactose, used in our study had any positive effect on lovastatin production. Generally, nitrogen sources (organic and inorganic) have a vital role in increasing lovastatin production. However, none of the nitrogen sources (organic and inorganic) tested with *Aspergillus spp* showed any effect on increasing lovastatin production.

CONCLUSION

The current investigation was mainly focused on the screening of various fungal species for lovastatin production under Liquid state fermentation. The isolated fungus *Aspergillus spp* showed the maximum yield of lovastatin. As optimum conditions of lovastatin production by *Aspergillus spp* using different Carbohydrates Source have been attained efficiently by HPLC, the Lovastatin yield (3.353 mg/g dry fermented matter) was achieved with the following optimized culture conditions (temp. of 28 °C; pH of 5.00; initial moisture content of 70% and incubation period of 12 days). The feasibility of Liquid state fermentation (LSF) as a promising technique in exploiting cheaply available fermentation media and employing HPLC as an optimization technique not only increases the yield but also results in economic lovastatin production.

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