Screening and Identification of Lovastatin producing endophytic fungi

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In

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Submitted By

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Certificate

This is to certify that the thesis entitled "**Screening and Identification of Lovastatin producing endophytic fungi**" being submitted by **Ms. Manpreet Kaur (Roll No-301401008)** in partial fulfillment of the requirements for the award of degree of master of science in biotechnology, Thapar University, Patiala is a bonafide work carried out under the esteemed supervision and conception of **Dr. Sanjai Saxena** and that no part of this thesis has been submitted for the award of any other degree.

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I hereby declare that the work being presented in the thesis entitled "**Screening and Identification of Lovastatin producing endophytic fungi**" in partial fulfilment of the requirements for the award of degree of Master of science, Department of Biotechnology, Thapar University, Patiala is my own laboratory work during the period of **January 2016 to June 2016**, under the conception and supervision of Dr. Sanjai Saxena, Professor, Department of Biotechnology, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

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I feel grateful while dedicating this thesis to my parents...

Thank you mom and dad for being there in my thick and thin! Regards… ☺

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Matre devo bhava, Pitr devo bhava, Guru devo bhava!

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Manpreet Kaur

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Abbreviations

S.No	Abbreviation	Full form
1.	ACN	Acetonitrile
2.	AD	Alzheimer's dementia
3.	COX	Cyclooxygenase
4.	DNA	Deoxyribonucleic acid
5.	dNTP	Deoxynucleotide triphosphate
6.	EA	Ethyl acetate
7.	EDTA	Ethylene diamine tetra acetic acid
8.	ITS	Internal transcribed spacer
9.	L	Litre
10.	M	Molar
11.	mAU	Milli-absorbance unit
12.	mM	Milli-molar
13.	mg	Milli-gram
14.	mL	Milli-litre
15.	mm	Milli-metre
16.	nm	Nanometer
17.	NO	Nitric oxide
18.	PCR	Polymerase Chain Reaction

19.	ppm	Parts per million
20.	µg	Microgram
21.	µL	Microlitre
22.	R_f	Retention factor
23.	rpm	Rotation per minute
24.	GLA	Grape leaves agar
25.	HPLC	High performance liquid chromatography
26.	MEA	Malt Extract Agar
27.	PDA	Potato Dextrose Agar
28.	TAE	Tris acetate EDTA
29.	TE	Tris EDTA
30.	TFA	Tri-fluro acetic acid
31.	TLC	Thin layer chromatography
32.	CVDs	Cardiovascular diseases
33.	CZ	Czapek Dox Agar
34.	CYA	Czapek Yeast Extract Agar
35.	YES	Yeast Extract Sucrose
36.	MEA	Malt Extract Agar
37.	ITS	Internal Transcribes Spacer
38.	HMG - CoA	3 – hydroxyl – 3 – methyl glutaryl coenzyme

Executive Summary

Hypercholesterolemia is a potent risk present in the world due to modern lifestyle. It is a silent killer which will claim nearly 3.5 million lives in upcoming decade according to WHO. Lovastatin also known as 'Merck's Mevacor' is an anti-cholesterol agent. Lovastatin blocks the cholesterol synthesis by acting as a competitive inhibitor of HMG – CoA reductase and thus inhibits the mevalonate pathway. However, due to limited availability of lovastatin, there is an utmost need to explore alternative natural sources to meet its scarcity.

Endophytic fungi is an endosymbiont which colonize the healthy living tissues of host plant asymptomatically. Endophytic fungiare considered to be ware houses of plethora of bioactive compounds which exhibits antimicrobial, antifungal and immunosuppressive activities. The present study was based upon screening and identification of Lovastatin producing endophytic fungi. Initially, Culture filtrates of 36 Cultures isolated from *Aegle marmelos*, *Cinnamomum sp.* were screened for Lovastatin production by Lactonization assay. Out of 36 cultures, #8 AMSTYEL and #1 CMLNEY showed potential lovastatin activity. Further, crude ethyl acetate extract was resolved into 6 bands in methanol: DCM solvent system. Band 6 of crude extract was found to be exhibiting same R_f value as that of Standard Lovastatin. The concentration and further confirmation of Lovastatin production was ascertained by using HPLC. The crude EA extract was showing a peak at 2.30 min comparable to single peak of standard Lovastatin at 2.29 min. The potential endophytic fungus, #8 AMSTYEL was identified as *Alternaria* sp. through classical tools. ITS region of approximately 550 bp was amplified and further speciation of fungi will be deduced after analyzing sequencing data of ITS region.

1. INTRODUCTION

In recent era of huge development, India has adapted to dramatic lifestyle changes with increased consumption of processed foods and moving to sedentary lifestyles in a little span of time than other nations. These drastic life style changes have increased the risks of devastating chronic non-communicable diseases like cardiovascular diseases (CVDs), coronary heart diseases (CHDs), diabetes and stroke in the last decade with greater contribution of CVDs (Chauhan et al 2015).

A total of 17.3 million mortality cases are reported per year due to CHD and this number will rise to > 23.6 million deaths by 2030 (www.heart.org). According to American Heart Association statistics, close to 0.79 million people lost their lives due to CHD, stroke and other heart ailments in 2011 (www.heart.org). Statins are biosynthesized by several filamentous fungi as secondary metabolites. They inhibit the formation of cholesterol by blocking the Mevalonate pathway. Statins are competitive inhibitors of HMG – CoA reductase. They block the active site of the enzyme, thus preventing it to act upon its substrate, HMG – CoA. Mevalonate pathway is blocked and thus cholesterol biosynthesis does not occur therefore, statins are treated as first choice of drug for treatment of Hypercholesterolemia.

Since the introduction of lovastatin in clinical use in 1987, the inhibitors of HMGR have proved remarkable success in clinical use as safe and effective drug, therefore now a days statins specially lovastatin are widely prescribed by doctors in cholesterol lowering therapy (Goswami et al. 2013; Istvan and Deisenhofer 2001)

Lovastatin is a very potent specific and competitive inhibitor of HMG – CoA (3–hydroxyl- 3- methyl glutaryl coenzyme A). Lovastatin is known by different names like Merck's Mevacor, Mevinolin and Monacolin K. It is a FDA approved drug which is biosynthesized by fungus. It is used in the treatment of hypercholesteremia, high levels of cholesterol in the blood. Nearly 17.5 billion people dies due to cardiovascular diseases (CVDs). Lovastatin is acting as a life saving drug, preventing excess cholesterol build up which further leads to artherosclerosis (Manzoni and Rollini 2002). Lovastatin is commercially available in white, crystalline powder form. It is sparingly soluble in ethanol, methanol and rarely soluble in water. Lovastain is produced as a predrug in a mixture of its lactone and beta – hydroxyl form. The lactone form is hydrolysed to beta – hydroxy form since it is the more active form (Samiee et al. 2003).

Lovastatin can be obtained from different sources like mushrooms, dark fermented tea and endophytes. Commercially, lovastatin is produced from endophytic microbes. Till date the highest yield of lovastatin has been reported from endophytic fungal species, *Aspergillus terreus* (Chan et al. 1983). During the biosynthesis of lovastatin, three to nine acetate units join together. Nine molecules of acetate give rise to monacolin L, which in turn is converted into monacolin J by hydroxylation. This monacolin J is further converted into lovastatin by subsequent steps.

In the studies done on *Aspergillus terreus*, atleast 18 different genes have been reported to be involved in the biosynthesis of lovastatin. Among these, the function of 6 genes have been established which are – lov A, lov B, lov C, lov D, lov E and lov F. However, Lov A is involved in the regulation of cytochrome P450 genes. Lov B, C and F are involved in the polyketide biosynthesis. Lov D is involved in the production of esters. Lov E is a transcription regulatory factor (Hutchinson et al. 2000).

Lovastatin has both its pros and cons. It is an effective treatment in the case of artherosclerosis as it prevents plaque progression and thrombus formation. It has been found to be effective in case of progressive renal diseases. It prevents cancer by inhibition of Ras farnesylation thus preventing proliferation and migration of cancer cells (Xia et al. 2001). Lovastatin has found another potential application in case of bone fracture. Lovastatin has been reported to stimulate bone formation both in vitro and in vivo in case of murine (Garett et al. 2007). Experimental evidences are still being collected for effect of lovastatin in case of Multiple sclerosis.

Endophytes are those fungi which colonise a healthy plant tissue without any apparent symptoms. They complete atleast one life cycle inside the plant tissue (Strobel et al. 1999). The world's billion dollar endophyte, *Pestalotiopsis microspora*, was discovered from Himalayan yew tree, *Taxus wallichiana*. This fungus produces anticancer drug – Paclitexel. Endophytes have potential benefits for humans as they are known to produce antidiabetic, anticancer, immunosuppressive compounds. Among different species of plants, a plethora of endophytes exist (Bacon and White, 2000). Endophytes develop inside plant tissue as an endosymbiont. They gain nutrition from plant but also provide various benefits to the plant. Enophytes help the plant in its root and shoot elongation. These help the plant against abiotic stresses and pest attack. Endophytes enhance the efficiency of a plant for nitrogen uptake.

Endophytic fungal genus like *Aspergillus, Penicillium, Monascus, Fusarium,* etc. are known to produce lovastatin , As endophytic fungi reside inside the host plant it mimics all the property of the host so it could be a potent source for production of lovastatin for commercial use. Therefore endophytes can be explored for novel biological active secondary metabolites. Hence, the present study was undertaken to screen endophytic fungi of medicinally important plants for probable production of lovastatin and related compounds.

2. REVIEW OF LITERATURE

2.1 Hypercholesterolemia – A major health ailment

According to a report by WHO, cardiovascular diseases are the cause of 7.5 million deaths annually. 45% of such deaths are caused due to coronary heart disease and nearly 51% by stroke. If this trend continues, approximately 25 million people will lose their life due to CVDs (Syarifah et al. 2014). Artherosclerosis develops due to high blood cholesterol levels. LDL – Low density lipoproteins also reported as bad cholesterol leads to excess cholesterol build up and plaque formation inside arteries. This leads to half – a – million deaths in a year (Dhar et al. 2014).

High cholesterol levels are the major risk factor in coronary heart diseases as reported by several clinical and nutritional studies. Major problem is that 50% of cholesterol in humans is derived by de novo synthesis. Statins are the ray of hope in countering this ailment by blocking the mevalonate pathway through which cholesterol is synthesized (Seydametova et al. 2015).

2.2 Statins

Statins are biosynthesized by fungal species as secondary metabolites. These are mostly produced by filamentous fungi, both Ascomycota and Basidiomycota. These act as competitive inhibitors of HMG – Co A reductase and inhibit the cholesterol production (Bizukojc and Ledakowicz, 2007). Statins have two classes – Natural statins and Synthetic statins. Natural statins are lovastatin and pravastatin, which are mevastatin derived (Alberts et al. 1980). Other natural statin is compactin. Synthetic statins include atorvastatin, fluvastatin and simvastatin . Simvastatin is derived from lovastatin so, several researchers term it as semi – lovastatin. Atorvastatin and fluvastatin are derived from mevalonate and pyridine, respectively (Manzoni and Rollini, 2002). Simvastatin is often known as semi – synthetic statin since its source is lovastatin, which is a natural statin.

2.2.1 History of statins

A Japanese microbiologist, Akira Endo provided the first insight into the world of statins. He isolated mevastatin from the culture of *Penicillium citrinum*, while looking forward to isolationof antimicrobial agents (Endo et al.1967a; Toberts 2003). At that time, microbes were known to produce compounds which help them defend against other microbes. Mevastatin was also

reported to be isolated by researchers at Beecham Laboratories, from the culture of *Penicillium brevicompactum* (Brown et al. 1986). During the period of 1976, Merck & Co. invested their interest and resources for the discovery of new statins. Lovastatin, initially known as mevinolin was isolated from fungus, *Aspergillus terreus* (Alberts et al. 1980; Manzoni and Rollini 2002; Endo 2004).

Animal and clinical safety trials were performed with lovastatin in April 1980. Lovastatin proved to be a promising drug in lowering LDL (Low density lipoproteins) in volunteers and no adverse effects were seen. But due to its close structural resemblance with compactin, clinical studies on lovastatin were suspended too. Compactin, also a statin was believed to cause severe animal toxicity. However, in 1983, Merck again initiated its efforts and by 1986, applied for regulatory approval of lovastatin. FDA approved lovastatin on August 31, 1987. Simvastatin and pravastatin were available for clinical use by 1990 (Endo and Hasumi, 1997). Rosuvastatin is recently synthesized statin and Astra Zeneca submitted it as NDA before FDI in 2001(Manzoni and Rollini, 2002).

2.2.2 Chemical structure of statins

All statins possess a similar chemical structure. In each statin there is a commom polyketide part, which is hydroxy – hexahydro naphthalene ring system (Manzoni and Rollini 2002). Attachment of different side chains to this ring gives birth to a new statin. Polyketides are chemically inert and due to their biological activity, they allow microbes to survive in hostile environments. Polyketides have different effects on different biological targets (Chakravarti and Sahai 2004).

2.2.3 Benefits and Risks of Statins

Statins inhibit the biosynthesis of sterols by competitively inhibiting the HMG – CoA reductase. Statins are known to reduce the intercellular cholesterol, thus upregulating the expression of LDL receptors in LDL cells. This leads to clearance of LDL from the bloodstream (Lange 2004). Statins have shown promising effects in decreasing oxidative stress and vascular inflammation. They increase the stability of artherosclerotic lesions, thus reducing the chances of myocardial infarction (Grines 2006). Statins improve the endothelial function and reduce the concenterations of Tumor necrosis factor in plasma (Chaves 2006). There are several risk factors involved in the use of statins. Continuous use of statins may lead to nausea, diarrhea, constipation and mild

gastro – intestinal disturbances. Statins may also lead to hepatotoxicity and fatigue. Allergic reaction s and stomach pain are some other adverse effects (Sullivan 2007; Goswami 2012).

2.3 Lovastatin – Merck's Mevacor

Lovastatin is a very potent specific and competitive inhibitor of HMG – CoA (3 – hydroxyl- 3-methyl glutaryl coenzyme A). Lovastatin is known by different names like Merck's Mevacor, Mevinolin and Monacolin K. Lovastatin is a FDA approved drug which is biosynthesized by fungus. It is used in the treatment of hypercholesteremia, high levels of cholesterol in the blood. Nearly 17.5 billion population dies due to cardiovascular diseases (CVDs). Lovastatin is acting as a life saving drug, preventing excess cholesterol build up which further leads to artherosclerosis (Manzoni and Rollini 2002).

2.3.1 Chemical structure and properties of lovastatin

In each statin there is a commom polyketide part, which is hydroxy – hexahydro naphthalene ring system (Manzoni and Rollini, 2002). Attachment of different side chains to this ring gives birth to a new statin. Chemically, lovastatin is defined as [(1S, 3R, 7R, 8As) – 8 – [2 – [(2R, 4R) – 4 – hydroxy – 6 – oxo – oxan – 2 – yl]ethyl] – 3,7 – dimethyl – 1,2,3,7,8,8a – hexahydronapthalen – 1- yl](2S) – 2methylbutanoate (IUPAC name). Lovastatin is commercially available in white, crystalline powder form. It is sparingly soluble in ethanol, methanol and rarely soluble in water. Lovastatin has empirical formula – $C_{24}H_{36}O_5$ and molecular weight of 404.55. Lovastain is produced as a predrug as a mixture of its lactone and beta – hydroxyl form. The lactone form is hydrolysed to beta – hydroxy form since it is the more active form (Samiee et al. 2003; Goswami 2012).

2.3.2 Sources of lovastatin

Lovastatin can be obtained from different sources like mushrooms, dark fermented tea and endophytes. Commercially, lovastatin is produced from endophytic microbes. Till date the highest yield of lovastatin has been reported from endophytic fungal species, *Aspergillus terreus* (Chan et al. 1983).Different microbes have been reported to produce lovastatin as secondary metabolite which are *Aspergillus terreus*, *Monascus* sp., *Aspergillus niger*, *Aspergillus flavus*, *Pencillium purpurogenum*, *Pleurotus* sp., *Trichoderma viride*,*Pemcillium* sp. (Lai et al. 2007; Pecyna and Bizukojc, 2011).

In commercial scenario, *Penicillium* sp., *Monascus ruber* and *Aspergillus terreus* are of substantial importance. *Aspergillus terreus*, which has played substantial role in the commercial production of lovastatin, is a filamentous fungi which belongs to class Ascomycetes (Subazini and Kumar, 2011).

2.3.3 Biosynthetic pathway of lovastatin

During the studies of biosynthetic pathway of lovastatin, it has been reported that Monacolin L and Monacolin J are the intermediate molecules (Endo et al. 1985). Initially monacolin L is produced from nine molecules of acetate. Monacolin J is produced from monacolin L by hydroxylation. This monacolin J via subsequent steps is converted into lovastatin (Kimura et al. 1990).

Further research on *Aspergillus terreus* suggested that biosynthesis of lovastatin initiates with acetate units which are linked to each other in head – to – tail arrangement to form two polyketide chains (Chan et al. 1983; Moore at al. 1985; Shiao and Don, 1987). In some statins, methyl group is present in the side chain, or at C6 derives from methionine. It is inserted in the structure before the rings are closed (Shiao and Don, 1987). Cyclization of main chain occurs or it is esterified at C8 by some side chains. Then, through aerobic oxidation, oxygen atoms are inserted in the main chain. Therefore, it has been established by research that lovastatin was derived from acetate through a polyketide biosynthesis pathway (Endo et al. 1985; Chakravarti and Sahai, 2004).

2.3.4 Clinical benefits and risks associated with lovastatin

Lovastatin has both clinical benefits and health risks associated with it. It is the proven drug in the treatment of hypercholesterolemia – excess concenteration of cholesterol in the bloodstream. It lowers the LDL concenteration and manages its faster clearance from bloodstream. Lovastatin has antiatherosclerotic effects since it improves endothelial function, modulates the inflammatory response and helps in the maintenance of plaque stability. Lovastatin has shown promising effects in preventing thrombus formation and also prevent plaque rupture (Palmer et al.1990). Lovastatin reduces LDL level which cause artherosclerosis and increase level of HDL and hence lesion formation is prevented (Goldberg et al. 1990). Lovastatin also reduces the chances of Alzeimer's disease in patients who are hypercholestrolemic (Ohm and Meske, 2006).

Lovastatin helps in fighting renal ailments (Buemi et al. 2002). Lovastatin inhibits the proliferation and migration of cancer cells by inhibiting Ras farnesylation. It is associated with reduction of proliferation and migration (Xia et al. 2001). Research is going on regarding the use of lovastatin in case of bone fractures. Lovastatin initiates bone formation in vitro and in vivo and helps in healing fractures, as shown in case of murines (Garett et al. 2007). Experimental evidences are still being collected for effect of lovastatin in case of Multiple sclerosis. Lovastatin can induce allergic reactions like rashes, itching, pale skin, difficulty in breathing in some people. It can also lead to dark urine, chest pain, muscle pain and swelling in severe cases. There are signs of liver damage, yellowing of skin, swelling of mouth and face, tenderness and weakness, pale stools and blistered or peeling skin (Omar et al. 2001; Guyton et al 2006).

2.3.5 Mode of action of lovastatin

Cholesterol biosynthesis occurs through the Mevalonate Pathway, where mevalonate is the building block. Lovastatin abrupts this cycle by countering the interaction of HMG- CoA reductase and HMG – CoA.

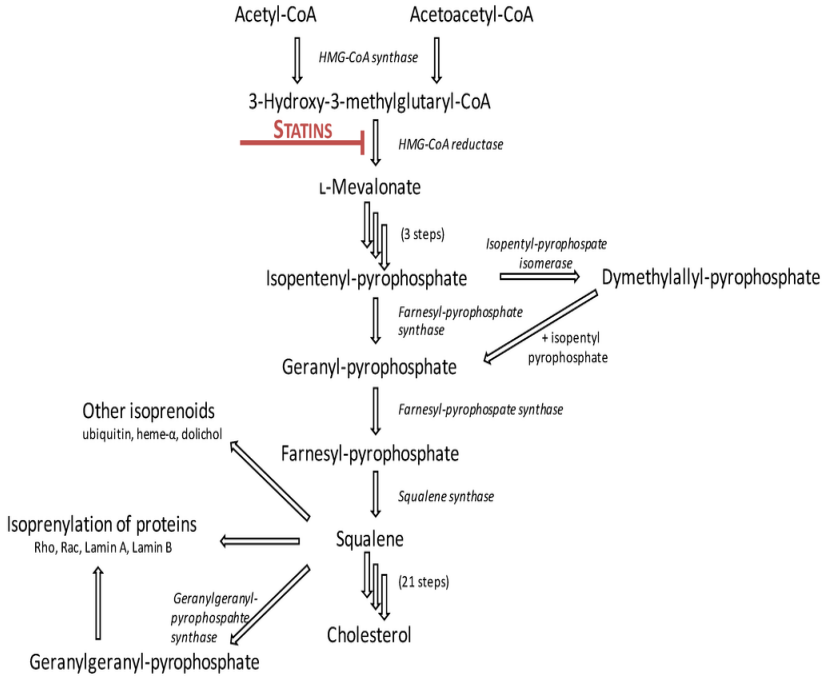

Figure 1 Lovastatin action in Mevalonate pathway (Basic principles of biochemistry, Lehninger, 2015, 6th edition, pg. 643-46)

Lovastatin acts as competitive inhibitor of HMG-CoA due to structural homology between the beta – hydroxyacid form of statins and HMG-CoA intermediate formed. Lovastatin is a mixture of lactone and its beta- hydroxyacid formin vivo (Samiee et al.2003). Lactone ring is hydrolysed into beta- hydroxyacid form, which is the active drug form of lovastatin. All statins except pravastatin, are produced as predrugs.

2.4 Endophytic fungi

Endophytes are those fungi which colonize a healthy plant tissue without any apparent symptoms. They complete atleast one life cycle inside the plant tissue (Strobel et al. 1999). The world's billion dollar endophyte, *Pestalotiopsis microspora*, was discovered from Himalayan yew tree, *Taxus wallichiana*. This fungus produces anticancer drug – Paclitexel. Endophytes have potential benefits for humans as they are known to produce antidiabetic, anticancer, immunosuppressive compounds. Among different species of plants, a plethora of endophytes exist (Bacon and White, 2000). Endophytic fungal genus like *Aspergillus, Penicillium, Monascus, Fusarium,* etc. are known to produce lovastatin (Subazini and Kumar, 2011). The highest yield obtained is from *Aspergillus terreus*. Enophytic producers of lovastatin have the gene cluster required for the biosynthesis of lovastatin. Lovastatin production can be screened from endophytic fungus by Lactonization test. Potential candidates are further screened through qualitative and quantitative analysis by TLC and HPLC respectively.

2.4.1 Isolation of endophytes

In order to isolate endophytes, plant tissue is dipped in 70% alcohol for 2 – 5 seconds. It is further treated with 0.5 – 3.5% sodium hypochlorite for 2 – 3 mins and rinsed with sterile water. Plant tissue is placed on nutrient medium and endophytes are allowed to grow. Endophytes peneterate plant tissue through their hyphae. The mode of reproduction is through spores which are dispersed by aerial route. Endophytes can also gain entry inside a plant tissue through natural cracks, wounds, air current, etc (Senthilkumar et al. 2000). An endophytic fungus must form a biological assosication with living plant while in its mycelia form, even if for some time. An endophytic fungus must form a biological assosication with living plant while in its mycelia form, even if for some time. An endophytic fungi should have hyphae in living plant tissue. (Bacon et al. 2000). Bleaching and staining technique can also be used for demonstration of

Microbial strain	Plant	Natural product	Biological activity	Reference
Acremonium zeae	*Zea maydis*	Pyrrocidine A, Pyrrocidine B	Antibacterial; Antifungal	Wicklow et al. 2005
Aspergillus clavatus	*Taxus mairei*	Brefeldin A	Antifungal; Antiviral; Anticancer	Wang et al. 2002
Aspergillus niger	*Cynodon dactylon*	Rubofusarin B	Cytotoxic; Xanthine oxidase inhibitor	Song et al. 2004
Cephalosporium sp.	*Trachelospermum jasminoides*	Graphislactone	Antioxidant; Free radical scavanger	Song et al. 2005
Cladosporium herbarum	*Cynodon dactylon*	Aspernigrin A; Rubrofusarin B	Cytotoxic; Xanthine oxidase inhibitor	Zhu et al. 2005
Dothiorella sp.	*Aegiceras corniculatum*	Cytosporone B; Dothiorelone A	Antifungal; Cytotoxic	Xu et al. 2004
Fusarium oxysporum	*Catharanthus roseus*	Vincristine	Anticancer	Zhang et al. 2004
Fusidium sp.	*Mentha avensis*	Fusidilactone A	Not specified	Krohn et al. 2002

Table 1: List of compounds isolated from endophytes

intracellular hyphae (Sathe and Raghukumar, 19991). Other method utilized for hyphae demonstration is acridine orange fluorescence microscopy (Senthilkumar et al. 2000).

2.4.3 Endophytes as endosymbiont

Being an endosymbiont, endophytic fungus provide several benefits to the plants, such as –

1. Increased shoot and/or root growth.
2. Enhanced systemic resistance to plant pathogens or insect pests.
3. Improved plant response to abiotic stress such as heat and salt.
4. Enhanced uptake of minerals possibly following solubilisation.
5. Enhanced nitrogen use efficiency (Strobel and Daisy, 2003).

2.5 Endophytes as source of lovastatin

Lovastatin is the secondary metabolite product of different endophytic fungi. These include *Aspergillus terreus,Apergillus niger*, *Penicillium* sp., *Aspergillus flavus*, *Penicillium purpurogenum*, *Pleurotus* sp., *Monascus* sp., *Trichoderma viride*. While from the viewpoint of commercial scenario, *Monascus ruber*, *Aspergillus terreus* and *Penicillium* sp are most substantial (Lai et al. 2007). The most prominent source utilized for the production of lovastatin is filamentous fungi, *Aspergillus terreus*. It belongs to class Ascomycetes of fungi (Pecyna and Bizukojc, 2011; Subazini and Kumar, 2011).

Submerged fermentation was carried out using *Aspergillus terreus* DRCC 122 in batch and fed – batch mode. Maltodextrin and corn steep liquor were added to medium as sources of carbon and nitrogen, respectively. Significant yield of lovastatin was reported (Kumar et al. 2000). Fermentation was carried out using *Aspergillus terreus* and glutamate and histidine were added as nitrogen sources. Results showed the highest biosynthesis level of lovastatin (Lopez et al. 2003).

Submerged fermentation was carried out using *Monascus purpureus* MTCC 369. Nutrient parameters were optimized and following composition of medium were used – 100g dextrose, 10g peptone, 2g potassium nitrate, 2g NH4 H2PO4, 0.5g MgSO4.7H20 and 0.1g CaCl2 in 1,000 ml distilled water (Sayyad et al. 2007). Twenty – three fungal species, which belonged to ten different genera were screened for the potential lovastatin producers on an oat meal medium. Among them, best lovastatin yield was given by *Aspergillus terreus*, followed by *Aspergillus flavus*. Though there was not much difference in the concenteration of lovastatin produced (Osman et al. 2011).

Siamak et al. (2003), while studying potential lovastatin producing candidates among endophytic fungi, screened nearly110 strains of atleast 22 genera and 50 species. Results showed *Aspergillus terreus* as best lovastatin producing species with production of 55mg/l of lovastatin. A concenteration of less than 20 mg/l was obtained from other screened strains such as *A.fischeri* (2mg/l), *A.flavus* 99mg/l) and *A.umbroscus* (14.1 mg/l).

According to a published research, 25 different fungus species were tested and results were found positive for one – third of them. The best lovastatin producer strain was *Aspergillus terreus*, with a concenteration of 84mg/l (Shindia 1997). Red mold rice was produced by co – culturing two endophytic fungus species, *Monascus purpureus* and *Monascus ruber*. When this fermented rice was consumed it lowerted total serum cholesterol levels and triglyceride levels as lovastatin was present in it. This showed that food grade endophytic fungi could be utilized for lovastatin production (Prasad et al. 2008).

Various research carried out on *Monascus* sp. Have shown it's a good lovastatin producer. This food grade fungal species was further utilized on rice and barley substrate in solid state fermentation. One *Monascus purpureus* culture and three *Monascus ruber* cultures were positive for lovastatin production (Upendra et al. 2013). Endophytic microfungi *Penicillium* species were isolated from soil in Pahang State of Malaysia. These were cultured on Potato Dextrose Agar and screened for the production of lovastatin. Researchers tested 54 different strains and among them four were positive for lovastatin production. The strain *Penicillium* ESF2M gave the highest yield of 20mg/l (Seydametova et al. 2012).

Results from different research have shown *Aspergillus terreus* to be the best lovastatin producer with the highest production levels of 84mg/l. Other potential candidates for lovastatin production are *Penicillium citrinum* and *Trichoderma viride* with concenterations of 61mg/l and 4.1mg/l (Shindia et al. 1997). Mangunwardoyo and Kusmana (2012) screened for potential lovastatin producing fungi among molds of *Aspergillus* from University of Indonesia Culture Collection. Among 40 different cultures screened, 18 showed positive results. Among the highest lovastatin producer was *Aspergillus flavus*.

Manzoni et al. (1999) studied various endophytic fungal species for lovastatin production. It was found that *Monascus* and *Aspergillus* were best lovastatin producers. The yield from a mutant *Monascus* strain *M.paxii* AM12M was 127mg/l and from that of *Aspergillus terreus* was

230mg/l. A research was carried out on *Fusarium* and it was concluded that it has PKS gene that is essential for lovastatin production (Raghunath et al. 2012).

2.6 Screening for potential lovastatin producers

The analysis of potential lovastatin producers was done through lactonization test. In this, after the production and filteration of culture, 1 ml of 1% trifluroacetic acid was added to 1 ml of supernatant. It was incubated for 10 mins during which hydroxyl acid form of lovastatin was lactonized. 1 ml of above solution was taken and diluted it 10 times. Further 1 ml was taken and diluted 10 times with acetonitrile. Absorbance was taken at 238 nm by UV – Visible spectrophotometer (Latha et al. 2015).

For the screening of lovastatin producing strains, submerged fermentation was carried. Cultures were filtered and supernatant was obtained. To 1 ml of supernatant, added 10 ml of 1% trifluroacetic acid. Absorbance was taken at 238 nm (Su et al. 2003). The solution was further concenterated and then diluted with acetonitrile. HPLC analysis was carried out (Sayyad et al. 2007).

Raghunath et al. (2012) carried out solid state fermentation of different fungal species for lovastatin production. Supernatant was obtained and lactonization test was carried out. 1 ml of supernatant was taken and 1 ml of 1% trifluroacetic acid was added to it. Incubation time of 10 mins was provided to the solution during which hydroxyl acid form of lovastatin was lactonized. 0.5 ml of this solution was taken and further diluted 10 times with methanol. By using UV – Visible spectrophotometer, absorbance was taken at 238 nm. Various species of endophytic fungi were cultured and fermentation was carried out. Supernatant was evaporated and then resuspended in pure acetonitrile. Sample was filtered first and its absorbance was carried out at 238 nm with the help of UV – Visible spectrophotometer (Seenivasan and Panda, 2015).

2.7 Extraction of potential lovastatin producers

Potential lovastatin producing endophytic fungi were cultured in Czapek Dox Broth for 48 hours at room temperature. Filteration was donea and the filterate was acidified using 2N sulphuric acid upto ph 3. Homogenized mycelium was added to it and mixed properly. Extraction was carried out using equal volumes of ethyl acetate atleast thrice. Extract was evaporated to obtain

lovastatin on which quantitative and qualitative analysis were carried out (Mangunwardoyo et al. 2012).

Osman et al. (2011) provided endophytic fungus a fermentation period of 7 days in a medium containing different waste products such as molasses, strawberry and apple waste, wheat bran and bagasse. After the incubation period, the contents were acidified to pH 3 using concenterated HCl. A mixture of ethyl acetate : cyclohexane (v/v) was added. The solution was cooled for 24 hours and centrifuged to separate aqueous and organic phases. Organic phase was evaporated in rotary evaporator and obtained compound was reconstituted in methanol. Lactonization test was carried out to obtain absorbance at 238 nm. Fungal cultured were inoculated in different media and provided with an incubation period of 7 – 10 days. After the incubation period, the extraction process was carried out. Broth was acidified with HCl or diluted sulphuric acid. Ethyl acetate was added and agitated at ambient temperature. This was done thrice and organic phase was separated. It was dried and reconstituted and absorbance was taken at 238 nm. The compound was quantitatively and qualitatively analyzed (Panda et al. 2008; Upendra et al. 2013).

Upendra et al. (2013) cultured fungal spores on a production medium of novel composition for 15 days at room temperature. The fermented culture and its productwas dried for 1 hour and extracted with ethyl acetate. This was done using a shaker incubator for 1 hour at 180 rpm. Supernatant was analyzed through qualitative and quantitative analysis for the presence of lovastatin.

Solvents of different polarity were used to determine the best extraction agent for recovery of lovastatin. Methanol, ethyl acetate, acetonitrile and butyl acetate were added to fermented substrate. Solution was centrifuged at 200 rpm for 2 hours. Supernatant was collected and lactonized. Absorbance was taken at 238 nm and compared to standard lovastatin. HPLC analysis was done (Syarifah et al. 2014).

Fungal cultures were inoculated in Soyabean meal medium and fermentation was carried out. Broth was filtered and acidified to pH 2. Ethyl acetate was added and solution was kept in rotator shaker at 200 rpm for 2 hours. Organic phasewas obtained and dried. It was reconstituted and sample was further analyzed (Chaynika and Srividya et al. 2014).

2.8 Qualitative analysis of lovastatin using TLC

TLC was used for the screening and qualitative analysis of lovastatin production. Organic phase was collected after the extraction of sample and it was further concenterated using block heater. Then it was applied to Merck silica gel plates $60F_{254}$. Dichloromethane : Ethyl acetate (70:30) (v/v) was used as mobile phase. Three lovastatin standards were loaded as reference for R_f comparison. Plates were observed at 254 nm under UV lamp and later developed in iodine vapors (Saimee et al. 2003). TLC plates were coated with silica gel (200 mesh) and were air dried. These plates were further activated in hot air oven for 2 hours at 120^0 C. A crude sample of concenteration 200 microlitre was spotted along with standard. Dichloromethane : Ethylacetate (70:30) (v/v) was used as the solvent system. Plates were developed in the mobile phase atleast thrice. R_f value was calculated at 238 nm and plates were developed in iodine vapors (Hajko and Wildman, 1993; Jaivel et al .2010).

Sreedevi K. et al. (2011) prepared silica gel coated TLC plates. The compound after extraction was acidified and concenterated and spotted on plates. Solvent system used was Dichloromethane : Ethylacetate (70:30). Standard was also spotted for R_f comparison. Separated bands were scrapped off into different vials to which ethylacetate was added. These were filtered and the compound was analyzed using UV spectrophotometer at a wavelength of 238 nm. The compound obtained after extraction was acidified and concenterated. The sample was spotted in triplicates along with the standard sample. Dichloromethane : Ethylcetate (70:30) was used as solvent system. R_f value was compared and plates were observed under UV light. The bands that had similar R_f value to that of standard were scrapped off and the compound was further analyzed quantitatively. Plates were developed in iodine vapors. Other solvent systems can also be used like Toulene : Ethanol (80:20), Ethylacetate : Hexane : Acetic acid (70:30:6) and Diclorometha n : Acetic acid (85:15) (Sree Devi K et al. 2011).

Fungal spores were inoculated in PDB for 2 weeks. Medium was filtered and the filterate was spotted on TLC plates. Standard was also spotted for R_f value comparison. The solvent system used was cyclohexane : chloroform : isopropanol (5:2:1). Spots were visualized under UV light at 254 nm and developed in iodine vapors (Palinaswamy et al. 2012). Silica gel coated TLC plates were formed. These were spotted with standard and sample. Solvent system used was toluene : ethanol (80:20). Plates were visualized with the help of hand held UV lamp (Srividya et al. 2014).

Lovastatin sample from selected molds was obtained. Silica gel F_{254} was coated on TLC plates. These plates were activated in hot air oven for 1 – 2 mins. 20 ml of crude and standard were spotted on TLC plates. The solvent system used was Dichloromethane : Ethylacetate (70:30) (v/v). R_f values were compared under UV wavelength of 254 nm (Dhar et al. 2015).

2.9 Quantitative analysis of lovastatin using HPLC

Lovastatin exists in two forms – lactone and beta – hydroxyacid form. The beta – hydroxyacid form of lovastatin can be quantified by HPLC. Rapid analysis of lovastatin is possible using beta – hydroxyacid form since it elutes earlier from the column than the lactone fraction. Another factor is the stability of the beta – hydroxyacid form. Ferron et al. (2005) performed HPLC of lovastatin using a Beckman Ultrasphere ODS (250 * 4.6 mm I.D., 5 micrometer support diameter) column and a diode array detector was used. Mobile phase used was acetonitrile : 0.1% phosphoric acid (60:40) (v/v). the flow rate of the eluent was maintained at 1.5 ml/min. Wavelength used for detection was 238 nm and the concenteration of sample injected was 20 microlitre.

The standard lovastatin and the sample were analyzed using HPLC. The compound after extraction with ethylacetate was reconstituted in equal amount of acetonitrile. Resultant compoundwas filtered and subjected to HPLC. A C18 column was used and the mobile phase used was Acetonitrile : 0.1% phosphoric acid (60:40) (v/v). sample injection volume was kept 20 microlitre and flow rate was maintained at 1.0 ml/min. Temperature was kept at 25^0 C and lambda max was 238 nm (Sree Devi K et al. 2011).

Mangunwardoyo et al. (2012) carried out HPLC Of 1 gram lovastatin sample which was dissolved in 2 ml acetonitrile and 0.1 ml of 0.1% phosphoric acid. Incubation period of 30 minutes was provided and centrifugation at the rate of 1500 rpm was carried out. C 18 column was used and 20 microlitre sample was injected. Eluent rate was kept at 1.5 ml/min. Solvent system used was acetonitrile : 0.1% phosphoric acid (60:40) (v/v). ultraviolet ray detector was detected and lambda max used was 235 nm.

HPLC was carried out on compound extracted using ethylacetate. C18 id Zorbax Eclipse Plus column was used and 5 microlitre of the sample was injected. Mobile phase used was acetonitrile and water (60:40, v/v). the flow rate was maintained at 0.8 ml/ min and 238 nm wavelength was

used for the conformation of lovastatin. Pure lovastatin obtained from Merck was used as standard (Seydametova et al. 20120.

Sayyad et al. (2007) carried out HPLC of lovastatin using C18 column of SHIMADZU, Japan. The concenteration of the injected sample was kept at 20 microlitre. The solvent system used was acetonitrle : water acidified to the concenteration of 0.1% with orthophosphoric acid (60:40, v/v). Flow rate was maintained at 1.5 ml/min and lambda max used for detection was 235 nm.

Syarifah et al. (2014) carried out HPLC of lovastatin in isocratic mode. HPLC column used was that of Waters, USA. C18 column was used and mobile phase used was acetonitrile : phosphoric acid (77:23, v/v). The flow rate was maintained at 1 ml/min and absorbance was noted at 238 nm. Retention time was compared to that of standard pure lovastatin.

Sreenivasan et al. (2015) performed gradient HPLC of lovastatin using C18 column from SHIMADZU, Japan. Photodiode array detector was used. Mobile phase used was acetonitrile : acidified water (60:40, v/v). 20 microlitre of the sample was injected and the flow rate was maintained at 1 ml/min. the wavelength used for detection was 238 nm.

2.10 Molecular identification and Morphological characterization

Seydametova et al. (2015) carried out DNA isolation of *Penicillium* sp. ESF19M strain using DNA isolation kit obtained from Norgen Biotek Corporation, Canada. DNA sample was diluted using molecular grade water and its concenteration wasdeterminedat 260 nm using Eppendorf Biophotometer Plus. DNA integrity was checked using Agarose gel electrophoresis of concenteration 1%. The ITS region ITS1 – 5.8S – ITS2 was amplified using forward primer ITS1 and reverse primer ITS4 (White et al. 1990).

Seydametova et al.(2012) inoculated fungal spores on different media to study their morphological characteristics. Media used were CZ, CYA. YES and MEA. Colony diameters and colors varied on different media. Many cultures failed to sporulate on CZ and YES media. Colonies on CYA medium were white to cream, yellow or dark green. On YES media, colors were white to dark green and pale colors. These studies were carried out in *Penicillium* sp isolated from soil.

Upendra et al. (2013) carried out morphological characterization of fungal species using properties like colony color, shape, size, margins elevation and growth rate and different

microscopic properties like conidial head, conidiophores, vesicle and conidia. These samples were grown and studied on PDA and maintained further on PDA slants.

Goswami et al. (2013) carried out the genomic isolation of strain that gave the maximum lovastatin yield using 18s rDNA technique. The 18s sequence was amplified using forward primer - 27f (50-AGA GTT TGA TCC TGG CTC AG-30) and reverse primer - 1492r (50-TAC GGT TAC CTT GTT ACG ACT T-30). The data was further analyzed and aligned to find the closest homolog of the fungal strain. Bhargavi et al. (2015) carried out RNA isolation of selected lovastatin producer endophytic fungal strains by TRIZOL and STE (Sodium chloride, Tris and EDTA method). Further cDNA was constructed by reverse transcription. RT – PCR amplification was carried out using forward and reverse primers.

3. Aim of the Study

This study was conducted to fulfill the following objectives –

1. Screening of endophytic fungal isolates for lovastatin production
2. Identification of lovastatin producing endophytic fungal species

4. Materials and Methods

4.1. Sub culturing and maintenance of pure cultures

This involved preparation of Potato Dextrose Agar (PDA) plates, procurement of endophytic cultures from pre-existing repository maintained by Dr. Sanjai Saxena, Professor, Thapar university, Patiala. Further, sub culturing of the endophytic fungal cultures on PDA plates and long term preservation of pure cultures. A standard Lovastatin producing fungal strain GG3F16 was provided by Dr. Syed Riyaz-Ul-Hassan, Senior Scientist, CSIR-IIIM, Jammu.

4.1.1. Preparation of Potato Dextrose Agar (PDA) Plates

39.0 g of PDA was dissolved in one liter double distilled water and its pH was maintained at 5.2. The prepared media was dispensed into 250 ml conical flasks and autoclaved at 121°C, 15 psi for 15 minutes. Further, 25 ml of the autoclaved PDA was poured into the media was poured into sterile 90 mm petri plates aseptically and was allowed to solidify at room temperature.

4.1.2 Procurement, sub-culturing and maintenance of endophytic isolates

A total of 36 endophytic fungi isolated from *Aegle marmelos*, *Cinnamomum malabaricum* and *Cinnamomum zeylanicium* were sub-cultured on to PDA plates under aseptic conditions and incubated at 26 ± 2°C for 7-10 days. The inoculated plates were regularly monitored for fungal growth. After the incubation period was over, fungal hyphae were picked up and cultured on fresh PDA plates. These were maintained for long term storage by culturing them in test tubes containing PDA slants having 20% glycerol. Glycerol is a cryoprotectant which protects the sample from injury caused due to excessive freezing. It also helps in preventing heat shock. Sub culturing was done after regular intervals of time. The endophytic cultures were preserved on PDA slants with 15 % glycerol for long term storage at 4°C.

4.2 Production of culture filtrate

All 37 endophytic cultures were subjected for production of filtrates. 5 mm mycelial discs were scraped out by sterile cork borer and inoculated into a flask containing 50 ml of pre-sterilized PDB medium. The flasks were incubated at 28˚C, 120 rpm for 10 days. After the incubation period was over, filtrate and mycelia was separated using Whatman filter paper no. 4. It was centrifuged at 10,000 rpm at 4˚C for 10 min.

4.3 Screening of endophytic fungi for the production of lovastatin

Lovastatin exists in two forms – lactone and β – hydroxyl acid. Lactonization is a process in which lactone form is converted into β – hydroxyl form since it is the most active form. So, for the screening of Lovastatin producers, lactonization test was carried out. Different concentrations of Lovastatin (1mg/ml, Sigma-Aldrich) ranging from 50µg to 300µg were prepared (Table 2) to draw standard curve. The test involved addition of 1.5 ml of each culture filtrate into a test tube followed by 1.5 ml of trifluoroacetic acid (TFA). Further, the tubes were incubated at 37˚C for 10 min. The absorbance of each sample was noted at 238 nm using UV–Visible spectrophotometer (Raghunath et al. 2012). All the test reactions were performed in triplicates.

Concentration (µg)	Volume of stock Lovastatin (µL)	Distilled water (ml)
50	75	1.425
100	150	1.350
150	225	1.275
200	300	1.200
250	375	1.125
300	450	1.050

Table 2 Different concentrations of standard Lovastatin

4.4 Solvent extraction

The culture filtrate of endophytic fungi exhibiting potent lovastatin production capacity was further subjected to liquid – liquid extraction with ethyl acetate. To the culture filtrate, ethyl acetate was added in ration of (1:2) and shaken vigorously. The organic layer was collected in crucible, dried with anhydrous sodium sulphate and evaporated to dryness using rotatory evaporator. A dried fraction of crude bioactive compound was obtained. This fraction was weighed and reconstituted in methanol. Qualitative and quantitative analysis of these fractions were further carried out (Chaynika and Srividya et al. 2014).

4.5 Qualitative analysis using Thin layer Chromatography

Qualitative analysis of crude bioactive compound was carried out using preparative TLC. Silica gel obtained from Merck was coated on TLC plates of dimensions 20 × 15 × 5 mm and a thickness of 0.5 mm. These plates were activated by incubating at 70 – 80^0 C for 2 hrs in a hot air oven. The samples were spotted at 1 cm above the edge of the plates using capillary tubes.TLC chamber was saturated with a solvent system of Methanol: DCM in different ratios for at-least 30 min. The TLC plate was kept in the saturated chamber by taking care that the spotted sample was just above the layer of solvent. The solvent front was observed carefully and allowed to reach at a desired level. After this, TLC plate was taken out and observed under UV chamber for the R_f value of compounds. Standard used was pure lovastatin (Sigma – Aldrich) and R_f values of standard and sample were compared (Dhar et al. 2015).

Retention factor is calculated as the distance moved by the solute to the distance moved by the solvent.

$$\text{Retention factor } (R_f \text{ Value}) = \frac{\text{Distance moved by the compound}}{\text{Distance moved by the solvent front}}$$

4.6 Quantitative analysis by HPLC

The quantitative analysis of crude bioactive compound was done through HPLC (High Performance Liquid Chromatography). The HPLC apparatus having 200– series pump was obtained from Perkin Elmer. About 20 µg of bioactive compound was dissolved in methanol and injected into the HPLC column. Isocratic mode of elution was applied with a solvent system of Acetonitrile : (0.1%) Ortho – phosphoric acid (70:30). The flow rate was maintained at 1 ml/min. In order to determine the retention time of standard lovastatin, different dilutions of pure lovastatin (Sigma – Aldrich) were prepared in HPLC grade methanol. These concentrations ranged from 0.1 to 1.0 mg/ml. Each concentration was injected in a volume of 20 µl into C18 (5µm) Reverse phase discovery column (Sigma Aldrich) with 4.6 mm Internal Diameter x 150 mm Length. The peak area vs. concentration data of the pure lovastatin used as a standard was used to obtain the concentration of lovastatin present in the crude bioactive compound (Mangunwardoyo et al. 2012).

4.7 Identification of lovastatin producing endophytic fungi

Tools of classical morphotaxonomy and molecular morphotaxonomy were employed to identify lovastatin producing endophytic fungi.

4.7.1 Classical Morphotaxonomy

The potential lovastatin producing endophytic fungi were studied for their morphological and microscopic properties. The culture was grown over Grass Leaf Agar (GLA) and Pine Leaf Agar (PLA) for 3-4 weeks at 28^0 C (Seydametova et al. 2012). Different morphological characteristics such as colony shape, color, diameter of colony, pigment production, growth rate were studied. For microscopic examination, a clean glass slide was taken and a drop of water was placed over it. By the help of fine needle, mycelial mass was taken and placed over the drop of water. It was teased properly using a fine tip needle and a drop of Lactophenol Cotton Blue (HiMedia) was used to stain it. A cover slip of dimensions 18 × 10 mm was used to cover it while avoiding the formation of air bubbles. The slide was further mounted with DPX and observed under 10X, 40X and 100X using binocular microscope (Nikon, Japan).

4.7.2 Molecular Identification of potential lovastatin producers

4.7.2.1 DNA isolation of endophytic fungi

Fungal cultures were grown over PDA for 4-5 days at 28°C. Extraction buffer was prepared using 50mM Tris pH 8.0, 50mM EDTA, 3% SDS. In brief, 0.5-1g of mycelium was grounded to very fine powder by using liquid nitrogen in pestle and mortar. Add 660-750 µl of extraction buffer and crush again. Transferred the contents to 1.5 ml micro-centrifuge tube and add 10µl of ß-mercaptoethanol, 4µl of Proteinase K. Vortexed and incubated at 65°C in water bath for one hour (mixing after every 15 mins). Spin at 10,000 rpm for 15 min to remove all cell debris. Further, Add 6µl of RNase and incubated at 37°C for 30 min. Protein was precipitated by adding equal volume of phenol: chloroform (1:1) and centrifuged at 12,000 rpm for 15 min (repeat this step thrice). Aqueous phase was pooled and 20µl of 3M sodium acetate was added for DNA condensation. Invert the tubes gently and incubated at 4°C overnight. On the next day, centrifuged at 12000 rpm for 15 min to obtain DNA pellet. Wash the pellet with 70 % cold ethanol. Centrifuged at 12000 rpm for 2 min. Decant off the supernatant and let it air dry.

Finally, dissolved the pellet in 20 µl of TE buffer. Agarose gel electrophoresis was done to perform the qualitative estimation of DNA.

4.7.2.2 Agarose gel electrophoresis

Agarose gel (0.8%) was prepared in 1X TAE buffer and 0.5 µg/ml of EtBr (visualization dye) was added to it. Thes gel was poured in pre-set glass plate and comb was inserted to make wells. The gel was allowed to solidify. After solidification of gel, samples were mixed with 6X loading dye and were loaded into wells. Gel was allowed to run at 60 V for 1 h. Further, the gel was observed under UV trans-illuminator. For imaging of DNA, Bio – Rad Gel Documentation System which uses 1 – D Quantity analysis software was used.

For the quantitative analysis of DNA, the absorbance of DNA was taken at 260 and 280 nm. 50 µg/ml of DNA sample is equal to 1O.D. The concenteration of DNA was calculated by following formula–

$$\text{Concentration } (\mu g/ml) = O.D_{260nm} \times 50\mu g/ml \times \text{Dilution factor}$$

The purity of the DNA sample was analyzed by taking ratio of O.D. at 260 and 280 nm. If the value comes below 1.6, the DNA is contaminated with RNA. If it is between 1.6 – 1.8, the DNA sample is pure. If the value comes above 1.8, the DNA sample is contaminated by protein.

4.7.2.3 PCR amplification of genomic DNA

Amplification of ITS1-5.8S-ITS2 rDNA sequence was carried out using universal primer pair i.e. ITS 1 and ITS 4 (White et al. 1990) synthesized by Integrated DNA Technologies (IDT), USA, in a Thermocycler (Verti 96 well Thermal cycler, Applied Biosystems). Amplification was performed in 25 µl of reaction mixture was containing 25ng of extracted fungal DNA, 0.8 µM of each primer (ITS1 and ITS4), 2.5mM of dNTP (Bangalore GeNei), 1.5 mM $MgCl_2$ (Bangalore GeNei), 1.5 U of Taq DNA Polymerase (Bangalore GeNei) in 10 X Taq buffer (Bangalore GeNei) (Table 3). The conditions for Thermal cycler consisted of initial denaturation at 96ºC for 5 min followed by 39 cycles of 95ºC for 1 min, 58ºC for 1.30 min, 72ºC for 1 min followed by final extension at 72ºC for 5 min. The PCR amplicons were examined using gel electrophoresis in a 1.5 % agarose gel at 40V for 1.30 hr. Gel imaging was performed under UV light in Bio-Rad Gel documentation System. The amplicon was sent for sequencing to Xcleris Labs Pvt. Ltd.

S.No	Reagents	Stock concentration	Quantity	Final concentration in 25µl
1.	Autoclaved distilled water	_	15µl	_
2.	Taq buffer	10X	2.5µl	1X
3.	dNTPs	2.5mM	2.0µl	0.2mM
4.	Primers	10µM	2.0µl	0.8µM
5.	Taq DNA Polymerase	3U/µl	0.5µl	1U
6.	Template DNA	25ng/µl	1.0µl	25ng

Table 3 Composition of PCR reaction mixture

5. RESULTS AND DISCUSSION

5.1 Culturing and sub – culturing of endophytic fungi

All the 36 procured cultures were isolated from medicinally important plant sources – *Aegle marmelos*, *Cinnamomum malabaricum* and *Cinnamomum zeylanicum*. *Aegle marmelos* belong to family Rutaceae while *Cinnamomum* belongs to Lauraceae. These cultures were regularly sub-cultured on to PDA plates and further preserved as PDA glycerol stocks (Figure 2). Through the research done in past few decades, it has come to our scientific knowledge that endophytes produce plethora of bioactive compounds. Compounds showing potent biological activity such as anticancer, antimicrobial, cytotoxic and immunosuppressant activity have been isolated from endophytes. Compounds isolated from endophytic fungi have been used for the treatment of malaria, warts, fever and renal cancer. Lovastatin can be isolated from different sources such as mushrooms and dark fermented tea. Endophytic fungi are the potent sources of lovastatin. Our goal is to find lovastatin producing endophytic strains of fungi.

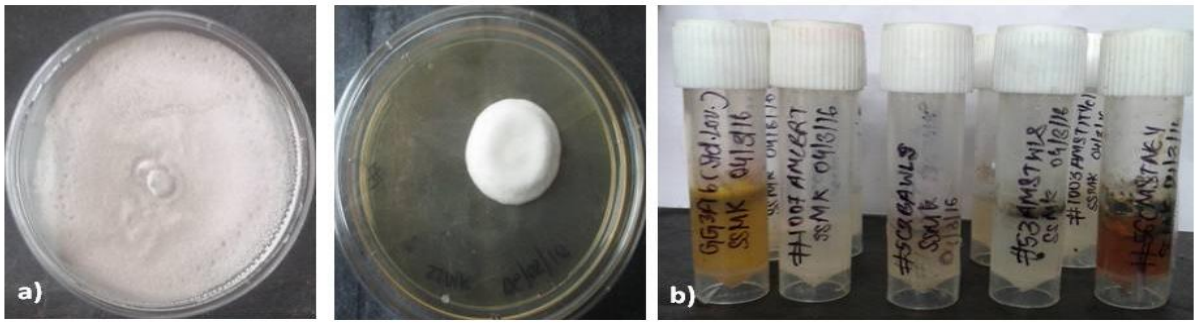

Figure 2 endophytic fungi under study on (a) PDA plates (b) preserved as PDA glycerol stocks

Out of 36 cultures, 27 were isolated from *Aegle marmelos*- 14 were from stem, 5 from stem internal tissue, 5 from leaves, 1 was from bark, 5 endophytic fungi were isolated from *Cinnamomum malabaricum*- 1 were from bark, 1 from leaf, 3 from stem. 1 endophyte from bark of *Cinnamomum camphora* and 2 from *Cinnamomum zeylanicum*- 1 from bark and other from stem internal tissue.

5.2 Production of culture filtrates of endophytes

All the endophytic cultures along with Lovastatin producing strain GG3F16 were subjected to secondary metabolite production. The growth rate of fungal cultures can be determined through biomass production. #8 AMSTYEL showed the highest biomass production while the cultures

with the lowest biomass production were #4 CMSTNEY and #2131 CZSTITG (Table 4). All the cultures were acidic in their pH except #1 AMSTYEL and #1003 AMSTITYEL which were slightly basic in their pH. #2131 CZSTITG gave maximum amount of culture filtrate while #1003 AMSTITYEL gave the lowest amount of culture filtrate (Figure 3).

Figure 3 showing culture filtrate production of endophytic fungi

S. No.	Culture Code	Dry weight of Biomass (g)	Volume of filtrate (ml)	pH
1.	#11 AMBAWLS	0.07	15.5	4.12
2.	#9 AMLBRT	0.09	17	5.10
3.	#1007 AMLBRT	0.12	15	6.57
4.	#1016 AMLBRT	0.04	13	6.28
5.	#6 AMLWLS	0.07	14.5	5.27
6.	#61 AMLWLS	0.16	17	3.70
7.	#1 AMSTYEL	0.13	6	7.09
8.	#3 AMSTYEL	0.15	16	6.39
9.	#4 AMSTYEL	0.13	16.5	5.00
10.	#5 AMSTYEL	0.09	16	5.60
11.	#6 AMSTYEL	0.15	15	4.61
12.	#7 AMSTYEL	0.10	18	5.85
13.	#8 AMSTYEL	0.17	13	6.58
14.	#9(b) AMSTYEL	0.09	18	5.42
15.	#11 AMSTYEL	0.07	17	5.94
16.	#17 AMSTYEL	0.08	16	6.22
17.	#22 AMSTYEL	0.08	17	5.71
18.	#23(b) AMSTYEL	0.08	14	4.53

19.	#1003 AMSTITYEL	0.05	3	7.23
20.	#1032 AMSTITYEL	0.09	17	4.41
21.	#1070 AMSTITYEL	0.11	17	5.72
22.	#1082 AMSTITYEL	0.04	17.5	4.30
23.	#1118 AMSTITYEL	0.06	17	4.22
24.	#28 AMSTWLS	0.09	10.5	4.15
25.	#47 AMSTWLS	0.07	17.5	5.95
26.	#53 AMSTWLS	0.03	16	6.05
27.	#59 AMSTWLS	0.11	16.5	6.35
28.	#1 CCBD	0.12	17	6.30
29.	#4 CMBABRT	0.11	17	6.45
30.	#1 CMLNEY	0.08	16	6.29
31.	#4 CMSTNEY	0.02	18	4.67
32.	#36 CMSTNEY	0.12	25	4.86
33.	#56 CMSTNEY	0.11	12	5.17
34.	#5 CZBAWLS	0.05	18	5.35
35.	#2131 CZSTITG	0.02	19	6.55
36.	#53 AMSTWLS	0.10	10	4.29

Table 4: Weight of biomass, volume of culture filtrate and pH of endophytic fungi

5.3 Preliminary screening of potential Lovastatin producer by lactonization test

Biochemical screening for potential lovastatin producers was done through Lactonization test. Lactonization test converts the lactone form of lovastatin into β – hydroxyl form which is the active form of the drug. Standard curve of Lovastatin (Sigma-aldrich) was plotted for concentration vs. absorbance (Figure 4). With increasing concentration of Lovastatin, the absorbance of β-hydroxyl form of Lovastatin was also increasing. Out of 35 cultures, 10 cultures along with GG3F16 were found to be positive for lovastatin production. #61AMLWLS, #6AMLWLS, #4CMBABRT and #8AMSYEL were found to be most potent Lovastatin producers followed by #1003 AMSTITYEL, #2131 CZSTITG, #1032 AMSTITYEL and

#1CMLNEY as compared to statndard Lovastatin producing fungi GG3F16 strain (Table 5). #11AMBAWLS and #1118AMSTITYEL were weak producers (Figure 5).

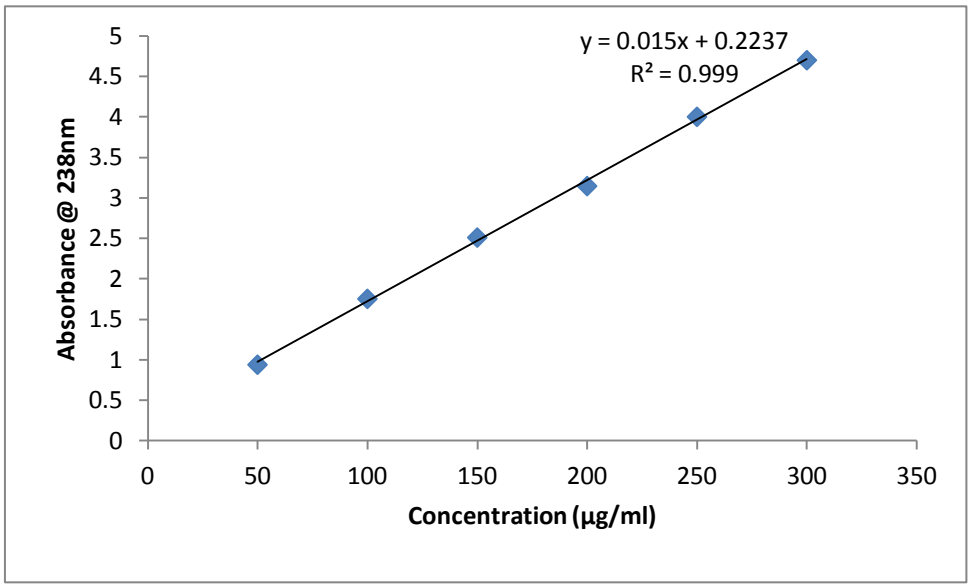

Figure 4 showing standard curve of pure Lovastatin by Lactonization test

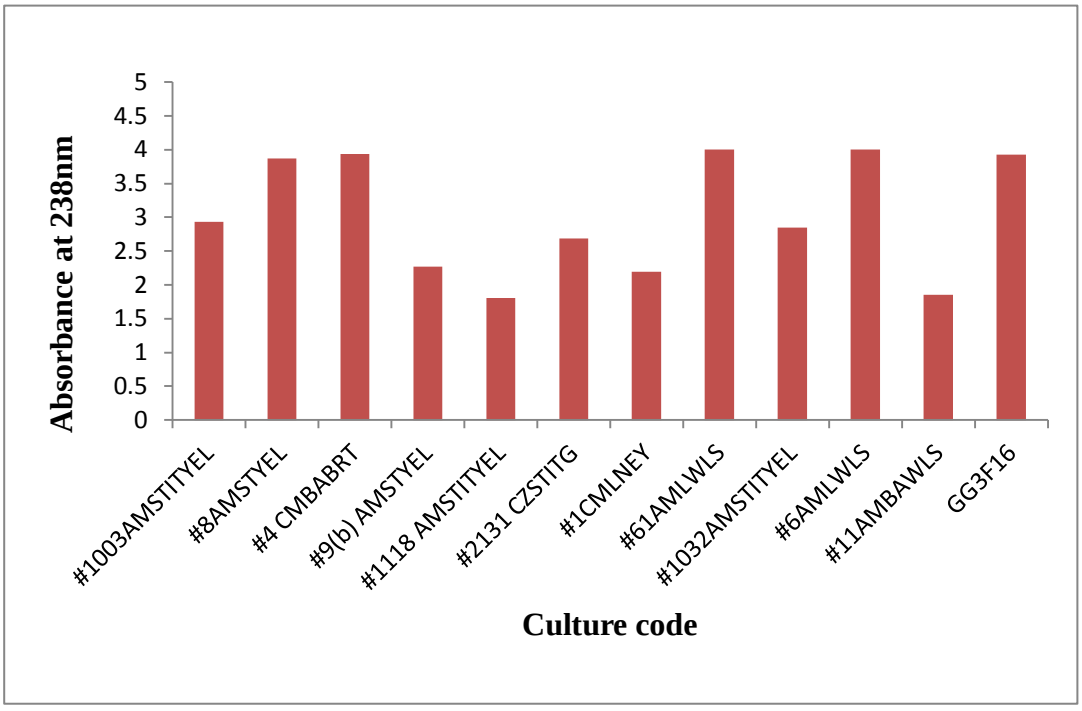

Figure 5 showing lactonization test of Lovastatin producing endophytic fungi

S.No	Culture Code	Plant part	Host Plant	Place of collection	Lactonization test
1.	#11AMBAWLS	Bark	*Aegle marmelos*	Wayanad, Kerala	+
2.	#9 AMLBRT	leaf	*Aegle marmelos*	BRT wildlife sanctuary	-
3.	#1007 AMLBRT	Leaf	*Aegle marmelos*	BRT wildlife sanctuary	-
4.	#1016 AMLBRT	Leaf	*Aegle marmelos*	BRT wildlife sanctuary	-
5.	#6 AMLWLS	Leaf	*Aegle marmelos*	Wayanad, Kerala	++
6.	#61 AMLWLS	Leaf	*Aegle marmelos*	Wayanad, Kerala	++
7.	#53AMSTWLS	Stem	*Aegle marmelos*	Wayanad, Kerala	-
8.	#1 AMSTYEL	Stem	*Aegle marmelos*	Yelundur,Karnataka	-
9.	#3 AMSTYEL	Stem	*Aegle marmelos*	Yelundur,Karnataka	-
10.	#4 AMSTYEL	Stem	*Aegle marmelos*	Yelundur,Karnataka	-
11.	#5 AMSTYEL	Stem	*Aegle marmelos*	Yelundur,Karnataka	-
12.	#6 AMSTYEL	Stem	*Aegle marmelos*	Yelundur,Karnataka	-
13.	#7 AMSTYEL	Stem	*Aegle marmelos*	Yelundur,Karnataka	-
14.	#8 AMSTYEL	Stem	*Aegle marmelos*	Yelundur,Karnataka	++
15.	#9(b) AMSTYEL	Stem	*Aegle marmelos*	Yelundur,Karnataka	+
16.	#11 AMSTYEL	Stem	*Aegle marmelos*	Yelundur,Karnataka	-
17.	#17 AMSTYEL	Stem	*Aegle marmelos*	Yelundur,Karnataka	-
18.	#22 AMSTYEL	Stem	*Aegle marmelos*	Yelundur,Karnataka	-
19.	#23(b) AMSTYEL	Stem	*Aegle marmelos*	Yelundur,Karnataka	-
20.	#1003 AMSTITYEL	Stem internal tissue	*Aegle marmelos*	Yelundur,Karnataka	+

21.	#1032 AMSTITYEL	Stem internal tissue	*Aegle marmelos*	Yelundur,Karnataka	+
22.	#1070 AMSTITYEL	Stem internal tissue	*Aegle marmelos*	Yelundur,Karnataka	-
23.	#1082 AMSTITYEL	Stem internal tissue	*Aegle marmelos*	Yelundur,Karnataka	-
24.	#1118 AMSTITYEL	Stem internal tissue	*Aegle marmelos*	Yelundur,Karnataka	+
25.	#28 AMSTWLS	Stem	*Aegle marmelos*	Wayanad, Kerala	-
26.	#47 AMSTWLS	Stem	*Aegle marmelos*	Wayanad, Kerala	-
27.	#53 AMSTWLS	Stem	*Aegle marmelos*	Wayanad, Kerala	-
28.	#59 AMSTWLS	Stem	*Aegle marmelos*	Wayanad, Kerala	-
29.	#1CCBD	Bark	*Cinnamomum camphora*	Darjeeling, West Bengal	-
30.	#4 CMBABRT	Bark	*Cinnamomum malabaricum*	BRT wildlife sanctuary	+
31.	#1 CMLNEY	Leaf	*Cinnamomum malabaricum*	Neyyar, Kerala	+
32.	#4 CMSTNEY	Stem	*Cinnamomum malabaricum*	Neyyar, Kerala	-
33.	#36 CMSTNEY	Stem	*Cinnamomum malabaricum*	Neyyar, Kerala	-
34.	#56 CMSTNEY	Stem	*Cinnamomum malabaricum*	Neyyar, Kerala	-
35.	#5CZBAWLS	Bark	*Cinnamomum malabaricum*	Wayanad, Kerala	-
36.	#2131 CZSTITG	Stem	*Cinnamomum zeylanicum*	Guwahati, Assam	+
37.	GG3F16	-	-	Jammu	++

Note: (+) and (-) indicates severity of Lovastatin production where (++) indicates good activity, (+) fair activity, (-) indicates no activity

Table 5 Preliminary screening of endophytic isolates under study for Lovastatin production by lactonization

5.4 Solvent extraction of lovastatin producing strains of endophytes

The cell free filtrates of positive Lovastatin producers were subjected to solvent extraction by ethyl acetate. The obtained bioactive compound further was reconstituted in methanol. #8 AMSTYEL (107 mg) gave the highest yield of bioactive compound and was lowest in case of #9 (b) AMSTYEL (1.2 mg) (Table 6). The bioactive compound of these selected cultures were again subjected to Lactonization test to identify potent producers. #8AMSTYEL and #1CMLNEY were found to be most potent lovastatin producers. The crude ethyl acetate residue of #8AMSTYEL produced dark red to orange colored compound while that of #1 CMLNEY produced dark yellowish colored compound. As the yield of #8AMSTYEL was found to be higher than #1CMLNEY so #8AMSTYEL was chosen for further analysis.

Culture Code	Yield of compound (mg)
#8AMSTYEL	107
#61 AMLWLS	75.7
#6 AMLWLS	1.2
#9(b) AMSTYEL	1.2
#4 CMBABRT	5.5
#1032 AMSTITYEL	1.8
#2131 CZSTITG	4.2
#1118 AMSTITYEL	14.7
#5 CZBAWLS	9.7

Table 6 Yield of bioactive compounds recovered from different cultures under study

5.5 Thin layer chromatography of potential lovastatin producer

The qualitative analysis of potential lovastatin producing culture #8 AMSTYEL was carried out through preparative thin layer chromatography (TLC). The crude bioactive residue of #8AMSTYEL was subjected to TLC by using different combination of solvents for achieving a good separation (Table 7). The optimized solvent combination at which the best separation was achieved was Methanol:Dichloromethane (50:50). The crude ethyl acetate extract of #8AMSTYEL was resolved into 6 bands. The Lovastatin (Sigma – Aldrich) was used as standard for comparison of R_f value. The standard Lovastatin exhibited same R_f value to as that of R_f value of Band 6 of crude extract of #8AMSTYEL (Figure 6)

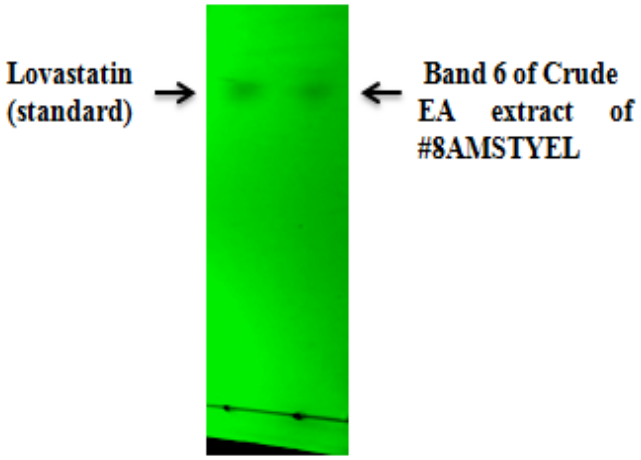

Figure 6 showing TLC of standard Lovastatin and crude EA extract of #8AMSTYEL under shortwave UV

S No.	Solvent system	Ratio used	Result
1.	Methanol : DCM	70 :30	No separation
2.	Methanol : DCM	60 : 40	Smear formation
3.	Methanol : DCM	65 : 35	Smear formation
4.	Methanol : DCM	40 : 60	3 bands observed
5.	Methanol : DCM	52 : 48	3 bands observed
6.	Methanol : DCM	55 : 45	1 band
7.	Methanol : DCM	49 : 51	4 bands observed
8.	Methanol : DCM	50 : 50	6 bands observed

Table 7 Separation of bioactive compound of #8 AMSTYEL using different combinations of solvent system

5.6 HPLC of ethyl acetate extract of lovastatin producing culture

The concentration of lovastatin in crude bioactive residue of #8AMSTYEL was determined by utilizing standard curve of pure Lovastatin drawn between peak area vs concentration of standard Lovastatin (Figure 7). Different concentrations of standard Lovastatin were injected into HPLC column (Table 8) and further retention time of standard lovastatin was compared with each peak of crude bioactive residue of #8AMSTYEL. The HPLC spectra of crude fraction showed two small peaks at retention time of 2.1 and 3.5 min, one medium peak at

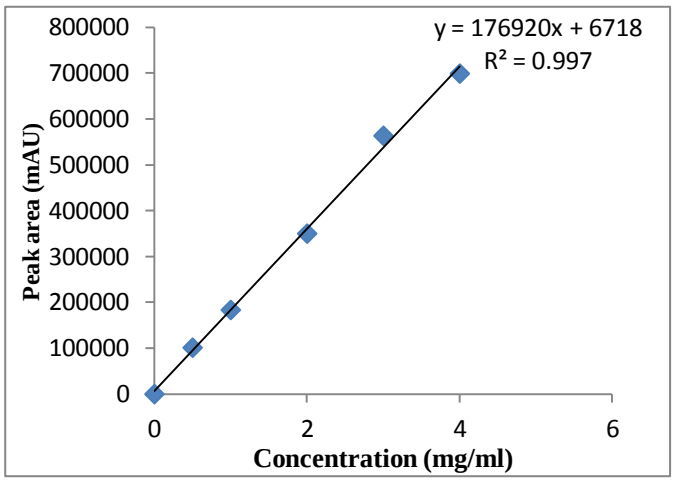

Figure 7 Standard curve of Lovastatin between peak area (mAU) and concentration (mg/ml)

Conc. (mg/ml)	Peak Area (mAU)	Retention Time (min)
0.5	101347.99	2.30
1	183608.49	2.27
2	350175.15	2.31
3	563608.49	2.30
4	699227.31	2.32

Table 8 Peak area and retention time of different concentrations of Lovastatin

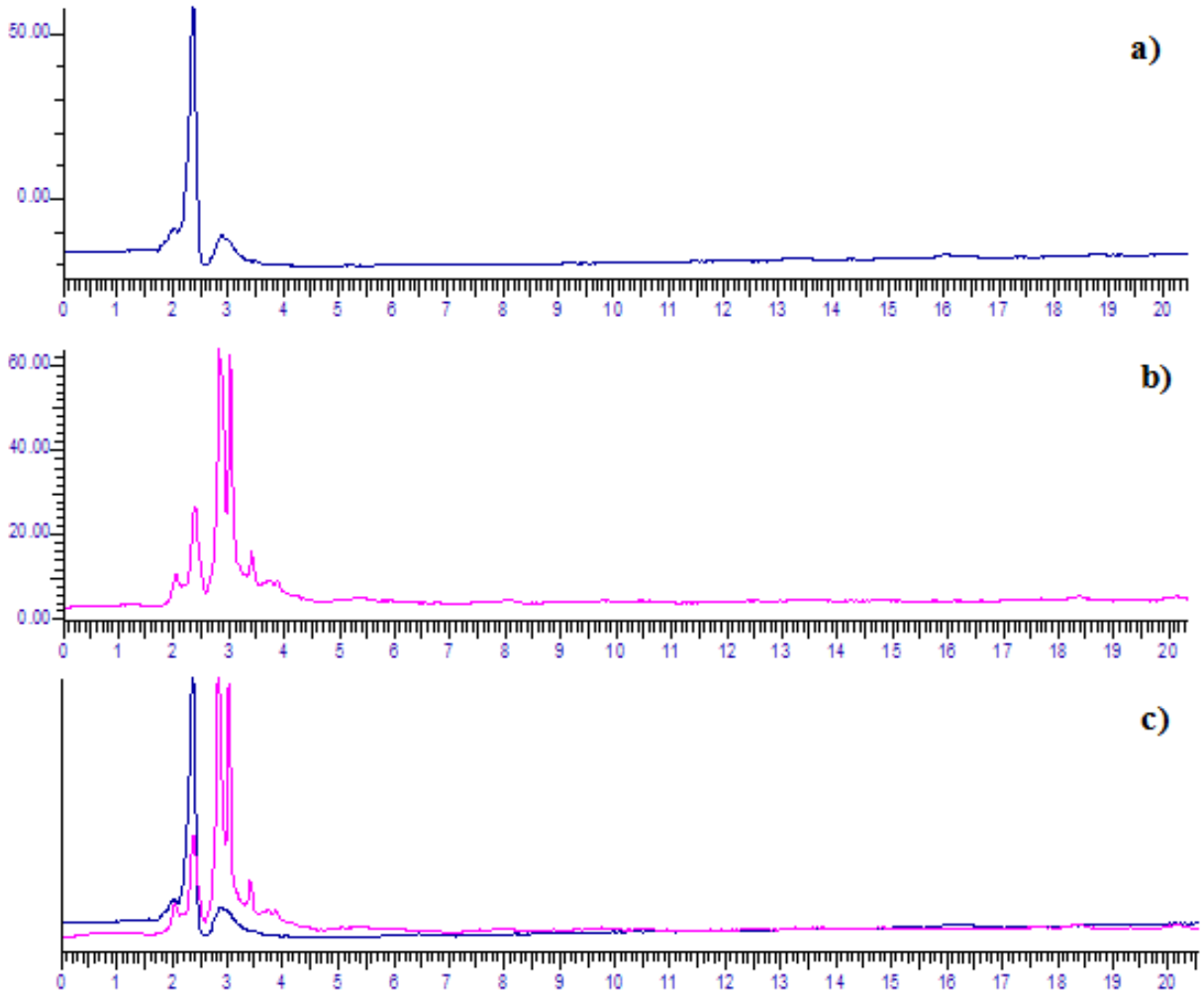

Figure 8 HPLC spectra of a) Standard Lovastatin b) crude ethyl acetate extract of #8AMSTYEL c) combined spectra of crude extract of #8AMSTYEL along with standard Lovastatin.

2.30 min and two prominent peaks at retention time of 2.9 and 3.1 min. However, standard Lovastatin showed a single prominent peak (Retention time- 2.32 min) (Figure 8).

5.7 Identification of lovastatin producing endophyte

The potential Lovastatin producing endophytic fungi was identified on the basis of classical as well as molecular taxonomic tools.

5.7.1 Classical Morphotaxonomy

Colony of #8AMSTEL over PDA medium was white in color from front and light brown in color from back, moderately growing with flat margins (Figure 9a). Over Pine Leaf Agar (PLA) and Grass leaf Agar (GLA), colony was brown in color from front and reverse side with flat margins. Its appearance was velvety to woolly. The fungus produces brown soluble pigment without any odour (Figure 9b-c).

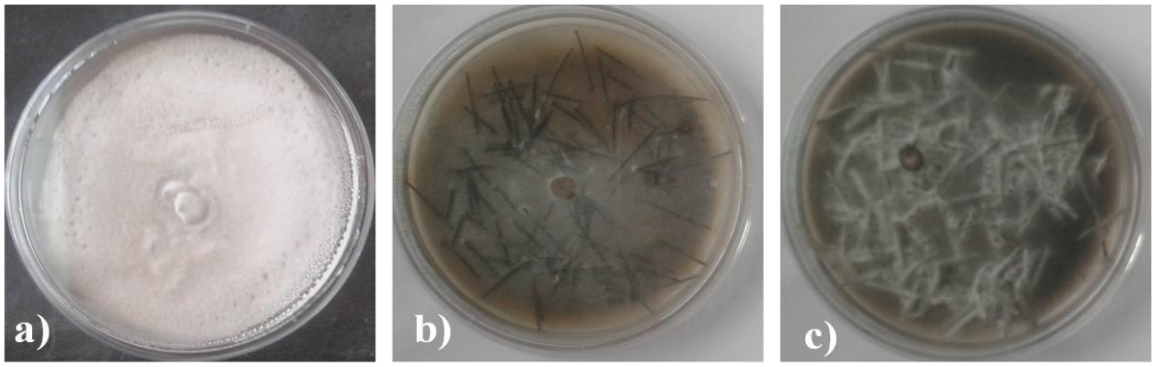

Figure 9 Colony morphology of #8AMSTYEL over different media a) PDA b) PLA, brown soluble pigment formation c) GLA,

Among microscopic characteristics, hyphae were thick, septate, multinucleate and branched. Conidia were brown colored, beak shaped, transverse and longitudinally septate. These were arranged in the chains of 6 – 18.

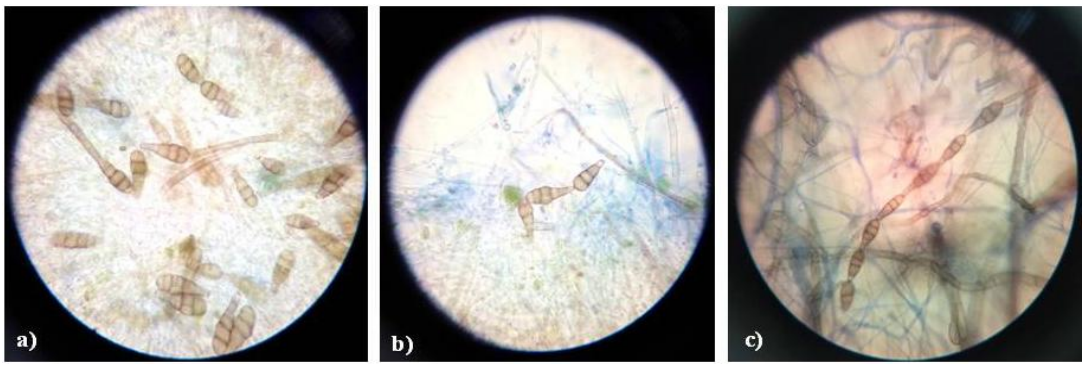

Figure 10 Microscopic features of #8AMSTYEL over GLA (a-b) brown colored conidia c) conidia arranged in chains

Hence, the potential endophytic fungi, #8AMSTYEL was tentatively identified as *Alternaria sp*.

5.7.2 Molecular identification

5.8.2.1 Genomic DNA isolation and PCR amplification

The genomic DNA isolation of the potent Lovastatin producing endophytic fungi, #8AMSTYEL was carried out (Figure 11a). The concentration of DNA was estimated by taking the absorbance at 260 nm and the amount was 35ng/µl by using formula

$$\text{Concentration } (\mu g/ml) = O.D_{260nm} \times 50\ \mu g/ml \times \text{Dilution factor}$$

The size of the PCR amplicon was found to be approximately 550 bp to 600 bp (Figure 11b). This size can be easily compared to the ITS region, which was amplified in order to characterize the fungi at molecular level.

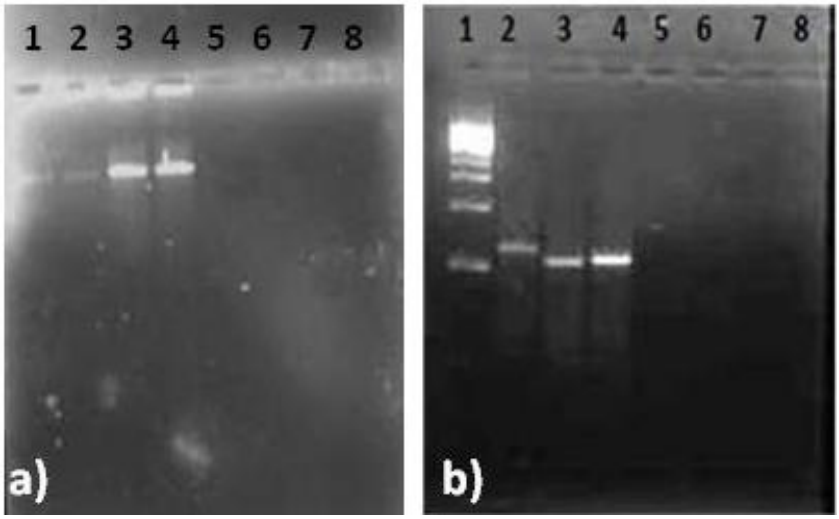

Figure 11 a) Genomic DNA isolation of #8AMSTYEL b) PCR amplicon of ITS region of #8AMSTYEL, lane 1: 500bp ladder; Lane 2-3: PCR amplicon of ITS region

Further, the sequencing of amplicon at Xcelris Labs, Ahmedabad, Gujarat is underway to ascertain its appropriate phylogenetic placement in *Alternaria* genus.

CONCLUSION

The present study concludes that #8AMSTYEL, isolated from *Aegle marmelos* is a potential Lovastatin producer. The presence of Lovastatin production was confirmed through TLC and HPLC results which comply with the standard pure lovastatin results.

The potential endophytic fungi, #8AMSTYEL was tentatively identified as *Alternaria sp.* through morphological and microscopic characteristics. The species level identification of #8AMSTYEL will be deduced after getting sequencing data of ITS region. For further confirmation of Lovastatin production by *Alternaria* sp (#8AMSTYEL), amplification of Lov E and LovF gene is under optimization. Further, purification of Lovastatin from crude EA extract #8 AMSTYEL needs to be carried out.

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