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RESEARCH ARTICLE

Studies on production of fungal secondary metabolite lovastatin

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Abstract

Natural products are important sources for new medicines and numerous bioactive natural compounds have been discovered from plants, microorganisms and marine sources. Approximately 60% of natural products or products derived and modified from such sources are currently being used in treatments of cancer and infectious diseases. Notable examples from fungal sources are multibillion dollar classes of antibiotics that include penicillins and cephalosporins, the important and widely used cholesterol-lowering agents lovastatin, compactin, and pravastatin, and the immunosuppressant cyclosporine-A. The market of drugs reached US$ 400 billion per year in 2005, with a steady yearly increase of more than 5%. Secondary metabolites from fungi and actinomycetes have provided us a huge array of important structures for various pharmaceutical purposes during the half of twentieth century.

Lovastatin is a cholesterol lowering agents produced as a secondary metabolite by a number of molds. *Aspergillus terreus* is widely used for the fermentation of lovastatin. Statins block the enzyme called hydroxyl-methyl glutaryl-coenzyme A reductase (HMG-CoA reductase) responsible for making cholesterol in the liver. The hypocholesterolemic effect of statins lies in the reduction of LDL (low-density lipoprotein) and increase in HDL (high-density lipoprotein). Of all the statins known today, only two are produced by fermentation de novo. These are lovastatin (also called mevinolin, monacolin-K or mevacor) and compactin (also called as mevastatin, or ML-236B). All the other statins are produced by chemically or enzymatically derivatising lovastatin or compactin.

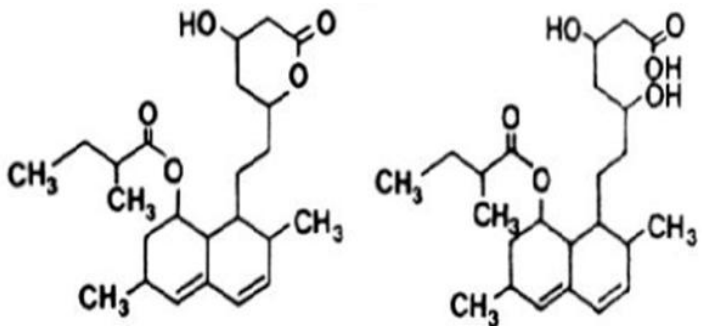
Introduction

Cholesterol is a soft, waxy natural substance found in the body. It plays an important role in maintaining normal body functions, however, high blood cholesterol leads to the development of atheroscleroris a type of heart disease causing death to more than a half million people annually. Low-density lipoprotein (LDL) and high-density lipoprotein (HDL) are types of cholesterol and concentration of these are involved in maintaining normal functioning of arteries. LDL or "bad cholesterol" is responsible for the buildup of cholesterol inside the artery walls. Alternatively, HDL or "good cholesterol" helps in removal of cholesterol from the blood and prevents arterial wall build up.

The primary role of the statins is to decrease LDL cholesterol. They work by slowing down HMG-CoA reductase, the enzyme responsible for cholesterol production. Additionally, statins increase the liver's ability to remove LDL cholesterol from the blood stream. Clinical trials have shown the significance effect of statins in the reduction of heart attack and stroke. Other important applications of statins are in the treatment of alzheimer's disease, renal disease and in cancer treatment etc. It has been observed that mean plasma cholesterol level decreased 19.4% from 294 to 237 mg/dl and LDL by 23.8% from 210 to 159 mg/dl with a statin therapy.

Hypercholesterolemia is considered an important risk factor in coronary artery disease. Thus the possibility of controlling de novo synthesis of endogenous cholesterol, which is nearly two thirds of total body cholesterol, represents an effective way of controlling plasma cholesterol levels. Statins, fungal secondary metabolites, selectively inhibit hydroxymethyl glutaryl-coenzyme A (HMG Co-A). Lovastatin was the first statin approved by United States Food and Drug Administration and made available in the market as anticholesterolemic drug (Tobert 1987). However, mevastatin was the first statin discovered in 1976 from a starin of *P. citrinum* and named ML-236B (Endo et al. 1976). Compactin, another antifungal metabolite was discovered during same time from a strain of *P. brevicompactum* (Brown et al. 1978).

Lovastatin (mevinolin, monacolin K, and Mevacor) is an inhibitor of 3-hydroxy-3methylglutaryl-coenzyme A reductase (HMG-CoA reductase), an enzyme that catalyzes the conversion of HMG-CoA to mevalonate. Mevalonate is a required building block for cholesterol biosynthesis and lovastatin interferes with its production by acting as a reversible competitive inhibitor for HMG-CoA, which binds to the HMG-CoA reductase. Lovastatin is inactive in lactone form which hydrolyzed to the biologically active form i.e. β-hydroxy acid in the body (Figure 1). All statins possess a common structure, a hexahydro naphthalene system and a β hydroxyl lactone. The studies on production of lovastatin were started since 1970's and it was observed that a wide range of statins were synthesized both as the final products and intermediates of secondary microbial metabolism, or as products of biotransformation process (Endo 1976 & 1979; Endo et al. 1986 and Manzoni and Rollini 2002). Several fungal genera including *Aspergillus*, *Penicillium*, *Monascus*, *Paecilomyces*, *Trichoderma*, *Scopolariopsis*, *Doratomyces*, *Pythium*, *Gymnoascus*, *Hypomyces*, *Phoma* and *Pleutotus* have been reported to produce Natural statins (Endo et al. 1985; Samiee et al. 2003; Cimerman et al. 1973; Shindia 1997; Komagata et al. 1989; Shiao and Don 1987; Miyake et al. 2006; Panda et al. 2008; Bizukojc and Stainslaw 2009; Cabral et al. 2010; Srinu et al. 2010).



A) Lactone form B) Acid form

Figure 1: Different forms of Lovastatin.

Biosynthetic pathways

From the various studies, it was demonstrated that monacolin L is the first to be synthesized from nine molecules of acetate which in turn converted to monacolin J by hydroxylation. Lovastatin is then derived from monacolin J. The investigation of the various pathways of synthesis of lovastatin was studied mainly in *Aspergillus terreus* strains employing labeled precursors showed that biosynthetic pathway starts from acetyl CoA, Malonyl CoA and S-adenosyl methionine by enzymatic hydroxylation and subsequent esterification. Various genes regulate the biosynthesis of lovastatin as shown in Figure 2 (González and Miranda 2010).

Screening of lovastain producing strains

A rapid screening method (bioassay) for the screening of lovastatin producing strains is developed by Kumar et al. (2000) and Ferron et al. (2005) using *Neurospora crassa* and *Candida albicans* as test organisms. A clear inhibition zone around the indicator organisms was the basis for the selection of suitable fungal strains for production of statins. Manzoni et al. (1999) screened several *Monascus* and *Aspergillus* strains towards statin production. They observed that of all the *Monascus* strains investigated *M. paxii* AM12M, a mutant produced 127 mg lovastatin/L and 53 mg of pravastatin/L at 21 days of fermentation using whole soybean flour medium. *A. terreus* BST yielded 230 mg lovastatin/L at 14 days of fermentation when defatted soybean flour was used in the medium. The production of

lovastatin by a wild strain of *A. terreus* was also evaluated by Szakacs et al. (1998) and they observed that isolate TUB F-514 produced lovastatin with better yield than strain ATCC 20542. In the present work, a number of molds were screened for biosynthesis of lovastatin using *N. crassa* and *C. albicans* as test organisms respectively and results are shown in Figure 3 and Figure 4 respectively.

Production process

Several fungal genera including *Aspergillus*, *Penicillium*, *Monascus*, *Paecilomyces*, *Trichoderma*, *Scopolariopsis*, *Doratomyces*, *Pythium*, *Gymnoascus*, *Hypomyces*, *Phoma* and *Pleutotus* are reported for the biosynthesis of lovastatin but, *Aspergillus terreus* is the most widely used mold for the production of lovastatin. Both submerged batch and solid state fermentation for production of this fungal secondary metabolite has been reported in the literature though the submerged batch fermentation process is maximally used with the cultivation time varied from 7 days to 21 days. The fermentation is carried out at 28 ^{o}C and pH 5.5-6.5 with glucose or lactose as main carbon sources. The industrial process for the production of lovastatin was set up in 1980 using an *A. terreus* strain (Mevacor, Merck). Seenivasan et al. (2008) analyzed different fermentation parameters such as culture homogeneity, effect of various carbon sources, pH, aeration, and agitator design for the production of lovastatin

The optimization of biosynthesis of this fungal metabolite was attempted by a number of workers but a clear methodology has not been concluded. Shan et al. (2005) investigated the effect of dissolved oxygen concentration on the production of lovastatin and observed that a increase of 20% in production of bioactive metabolite at a DO concentration of 20%. They have also studied the production by manipulating the pH of fermentation broth. Casas et al. (2005) studied the effect of speed of agitation and aeration on the fungal pellet morphology, broth rheology and lovastatin titre and noticed that the pellet diameter of approximately 2500 micron produced high concentration of lovastatin. A fed batch process for the formation of lovastatin was also studied by Novak et al. (1997) which showed 37% increase in the overall productivity. Solid state fermentation for production of lovastatin was also investigated using agricultural residues that resulted in higher yield with better product stability. Various factors like moisture content, pH, incubation temperature, inoculums size etc were optimized as different parameters for maximum productivity in solid state fermentation (Prabhakar et al. 2012).

The effect of lactose and glucose on the production of lovastatin by *A. terrues* ATCC 20542 was carried out by Lai et al. (2007) and they reported a 5 fold increase in production using lactose in the fermentation medium. Solid state fermentations were also performed for the biosynthesis of lovastatin. *Aspergillus flavipes* BICC 5174 was grown on solid state substrates (wheat bran, bagasse, barley, gram bran, soyabean meal, orange and pineapple fruit wastes) and produced 13.49 mg of lovastatin /g dry solid waste after 6 days of fermentation (Valera et al. 2005). Similarly, Lingappa and Vivek (2005) have described the production of lovastatin under solid state fermentation using an isolated strain of *A. Terreus* KLVB 28 with carob pods as substrate. The maximum yield of lovastatin produced was 220 microgram per gram of dry weight. Barrios-Gonzalez et al. (2008) studied the production of lovastatin in both solid state and liquid submerged fermentation using *A. terreus* TUB F-514 and found that the productivity in solid state fermentation is much higher than the submerged fermentation. As for as large scale production of lovastatin is concerned, we found most of production methodology and processes are either abstractive or covered by patents.

Hutchinson et al. (2000) described the importance of polyketides and related regulatory enzymes during the biosynthesis of lovastatin in *Aspergillus terreus*. The production of biomass and lovastatin by spore-initiated submerged fermentations of *A. terreus* ATCC 20542 showed an increase of 52% in production when the spore used was of the age in between 9-16 days (Rodriguez et al. 2006). A chemically defined medium was used to investigate the productivity of lovastatin by *A. terreus* (Hajjaj et al. 2001) and it was found an increase of three fold higher specific productivity as compared to production in complex medium with glucose, peptonized milk and yeast extract. Table 1 summarizes the list of different molds and some of the process parameters for maximum production of lovastatin.

Extraction of Lovastatin

Several methods have been described for extraction of statins from the fermentation broth. Before the extraction and recovery of the product, the pH of the broth is adjusted to 3 with dilute hydrochloric acid or sulfuric acid or trifluro acetic acid. The broth is then placed under agitation at ambient temperature or at high temperature for a fixed time interval in presence of equal volume of ethyl acetate. A combination of other solvents has also been attempted, but it was found that ethyl acetate was the most suitable solvent for maximum recovery of lovastatin. Finally, the organic phase was separated and dried (Panda et al. 2008; Upendra et al. 2013; Osman et al. 2011). The identification of compound is evaluated by spectrophometrically or by Thin Layer Chromatography. The λ max for

standard as well as for lovastatin was observed at 238 nm. The Rf value for standard and product was noticed at approximately 0.55 (Figure 5). The high pressure liquid chromatography is being used for quantification of the statins. Infra-Red and Mass spectrometry have been used for characterization of lovastatin. A summary of extraction process is presented in Figure 6.

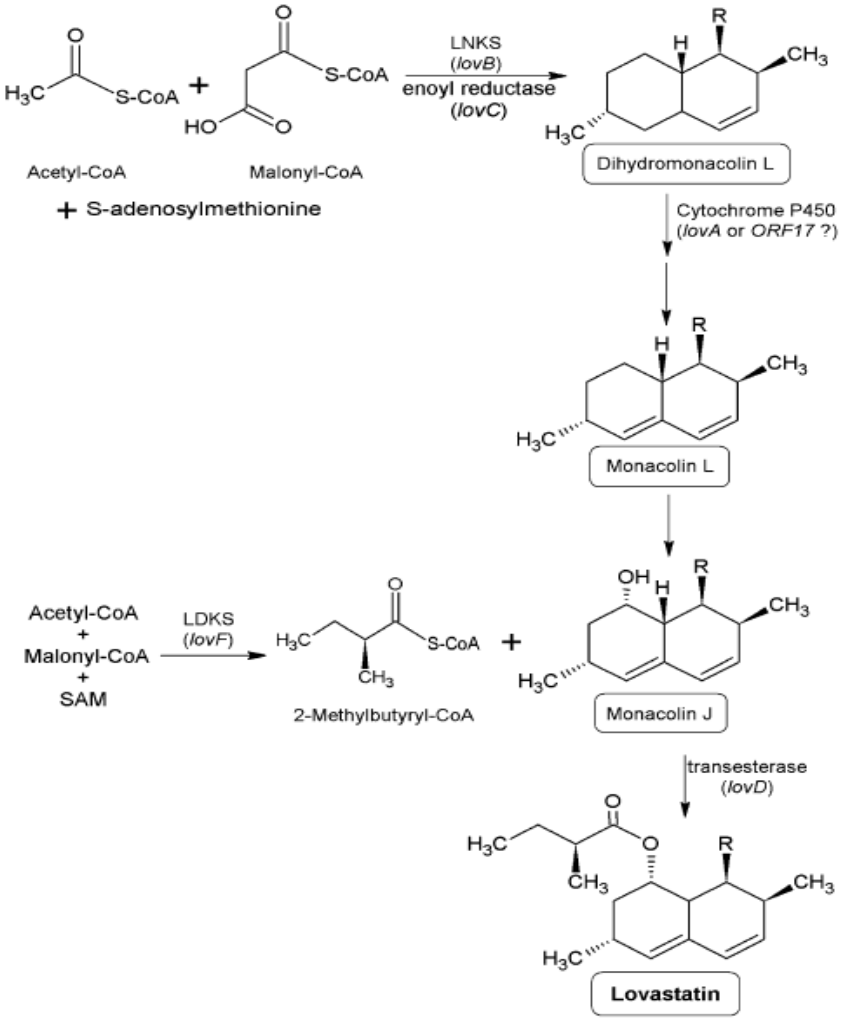
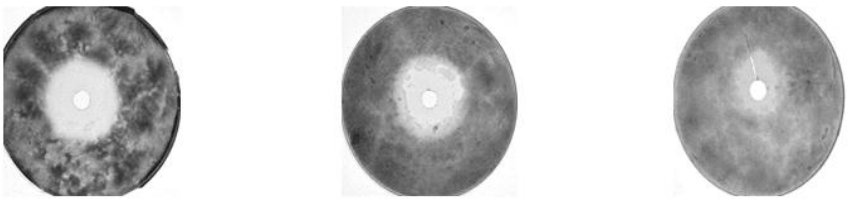


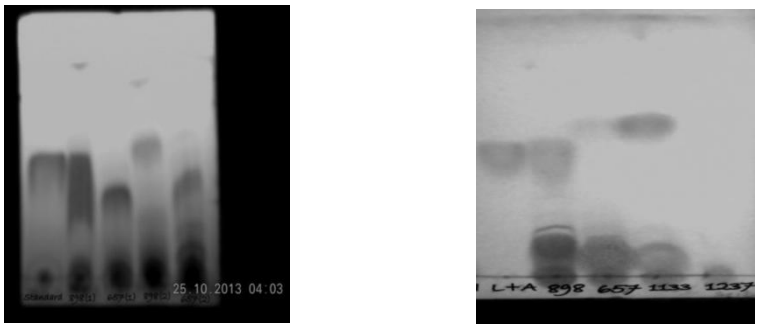
Figure 2: Lovastatin biosynthetic pathways, related enzymes and their encoded genes.



(a) *Aspergillus parasiticum* NCIM 898 **(b)** *Aspergillus terreus* NCIM 657 **(c)** *Phoma exima* NCIM 1237
Figure 3 (a-c): Zone of inhibition of lovastatin produced from different cultures against test organism *Neurospora crassa* NCIM 863.

(a) *Aspergillus parasiticum* NCIM 898 **(b)** *Aspergillus terreus* NCIM 657 **(c)** *Pleurotus ostreatus* NCIM 1200

Figure 4 (a-c): Zone of inhibition of lovastatin produced from different cultures against test organism *Candida albicans* NCIM 3471.



(a) (b)

Figure 5 (a-b): TLC chromatogram of standard as well the lovastatin produced from different molds.

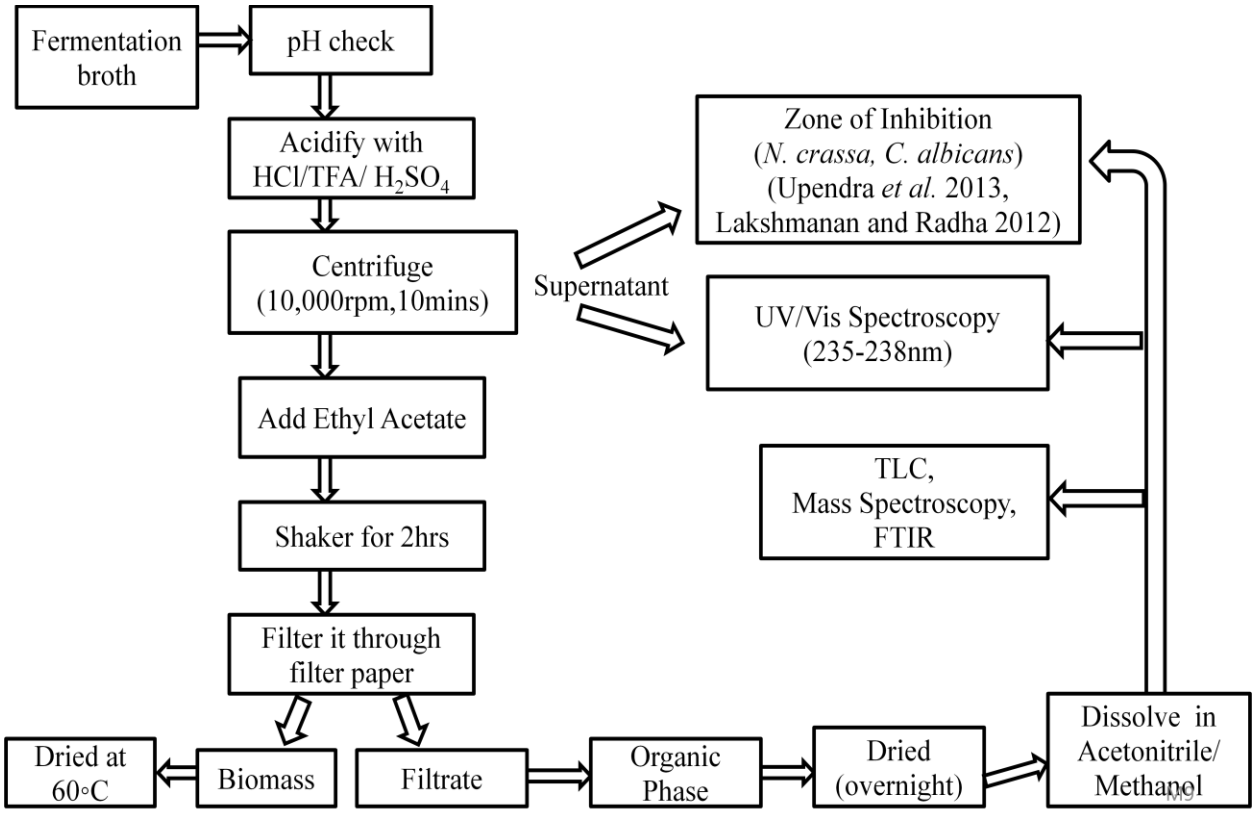


Figure 6: A schematic representation of extraction of lovastatin.

Table 1: List of certain molds and optimal conditions for lovastatin production.

S. No.	Fungi	Temp (o C)	pH	Carbon sources	Nitrogen sources	References
1	*Aspergillus terreus*	28	6.5	Lactose, Glucose, Sucrose	Corn steep Liquor and Corn meal	Szakacs *et al*. (1998)
2	*Aspergillus terreus* ATCC 20542	28	6.5	Lactose	Soybean meal	Casas *et al*. (2005)
3	*Monascus ruber* KFRI & CTCCCAS strains	30	5.0	Glucose	Yeast extract	Xu *et al*. *(*2005)
4	*Aspergillus terreus* ATCC 20542	28	6.5	Lactose	Soybean meal	Rodrıguez *et al*. (2006)
5	*P. ostreatus* PLUBB-127	28	6.5	Glucose	Yeast extract	Alarcon and Aguila (2005)
6	*Aspergillus terreus* NRRL 255	28	6.0	Glucose	Soybean meal	Gupta *et al*. (2009)
7	*Aspergillus terreus* LA414	28	6.5	Glycerol, Glucose, Sucrose, Lactose, Soluble starch	Yeast extract	Zhihua *et al*. (2009)
8	*Aspergillus terreus* CA99	28	6.0	Glycerol	Corn meal	Li *et al*. (2011**)**

Conclusions

Lovastatin acts as cholesterol lowering agent by inhibiting HMG-CoA reductase competitively. It decreases LDL level more than other cholesterol lowering drugs. A number of fungal strains are being used for the biosynthesis of different statins, though a complete production process needs to be thoroughly investigated. The most important parameters for the biosynthesis of lovastatin are the selection of carbon sources which depends on the producing strain. Also, the mode of cultural conditions play crucial role in the productivity. Statins are now prescribed for the treatment of many diseases like Alzheimer's disease, renal disease treatment, cancer treatment, bone fracture treatment and also as immunosuppressant.

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