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OPTIMISED PRODUCTION OF LOVASTATIN THROUGH SOLID STATE FERMENTATION BY ENDOPHYTIC FUNGI

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ABSTRACT

Cardiovascular diseases, the main cause of death in the society imply a strong correlation between enhanced blood cholesterol levels. 3-Hydroxy-3-methylglutaryl–coenzyme A (HMG–CoA) reductase catalyzes the conversion of HMG–CoA to mevalonic acid, plays a significant role in cholesterol synthesis. Fungi are the important sources for the production of lovastatin, a rate limiting step of cholesterol biosynthesis. In this study, endophytic fungal strain *Aspergillus niger* was tested for its ability to produce lovastatin by solid-state fermentation (SSF). Various substrates were used to evaluate the ability of *A. niger* to produce lovastatin. The organism produced high levels (1.5 mg/g of dry substrate) of lovastatin under optimized culture conditions on 8th day of incubation at an optimum pH 6.5, temperature 28 °C, moisture content 65%, particle size of 0.6 to 0.4 mm and bed depth 3 cm by SSF. The yields obtained in the present study would have to be further increased for its industrial importance but it has proved the feasibility of solid state fermentation as a promising technique in exploiting cheaply available agro-residual wastes as substrates for the large-scale production of microbial metabolites of biotechnological importance ultimately leading to an effective solid waste management.

KEYWORDS: **Lovastatin, SSF, *Aspergillus niger*, optimisation.**

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INTRODUCTION

Hypercholesterolemia, one of the leading risk factors for atherosclerosis and coronary heart disease, is a major problem worldwide [1]. Different drugs are available on the market for treatment of familiar hypercholesterolemia, of which the statins are frequently prescribed. Nowadays, lovastatin and its semisynthetic derivatives are very important drugs since the relatively high mortality of heart disease.

Statins (Lovastatin) are found to be an inhibitor of the lovastatin hydroxymethylglutaryl coenzyme A (HMGCoA) reductase that catalyzes the reduction of HMG-CoA to mevalonate during synthesis of cholesterol [2]. More efficient processes have been documented to increase lovastatin production [3,4]. The statins are originally a group of secondary metabolites isolated from fungi, such as lovastatin from *Aspergillus terreus* [3] and mevastatin from *Penicillium citrinium* [5].

Filamentous fungi synthesize many secondary metabolites with complex chemical structure via the polyketide pathway. Secondary metabolism of microorganisms is a part of their normal maturation processes [4]. The design of fermentation media is critical, especially when the products are secondary metabolites. Although major improvements are generally ascribed to the development of superior strains, nutrient supplies also affect cellular productivity.

In the past decades, lovastatin is mainly produced by submerged fermentation of *A. terreus* [6, 7]. As lovastatin is an intracellular product, and secondary metabolite of fungi, it is mostly accumulated in the mycelia. The major drawback in the submerged fermentation is that its yield is proportional to the biomass, with the high cell density causing the increase of the fermentation broth viscosity and the complexity in stirring and oxygen mass transfer. An alternative strategy for submerged fermentation is the SSF (solid state fermentation) process, which offers a good environment for fungi to grow, therefore high mycelia density and high lovastatin production can be expected.

With the emergence of new applications in clinical use for statins, researchers are actively trying to find new microbial strains capable of producing novel statins. The aim of this work was to screen the endophytic fungi for lovastatin production and to evaluate the feasibility of SSF process for optimised lovastatin production by *A. niger.*

MATERIALS AND METHODS

Microorganism and culture maintenance conditions

Endophytic fungi were isolated from *T. baccata* tissues obtained from central Himalayas, India. The plant parts were sterilized with Tween-80 for an hour. Tissue obtained was cut into small pieces (0.5 × 0.5 cm), treated with 70 % (v/v) ethanol for 15 seconds. These sterilized fragments were then treated with sodium hypochlorite for 15 mins. Treated tissue was placed on potato dextrose agar (PDA) amended with chloramphenicol 150 mg/L to decrease the amount of bacterial contamination. After several days, fungi were observed, and individual hyphal tips of the various fungi were removed and placed on a new PDA medium, and incubated at 25 °C for at least 2 weeks [8]. Based on the colony morphology and cultural characteristics, the fungus isolated was identified as *Aspergillus niger.*

Inoculum preparation

The spore suspension was prepared by adding 5 mL of suspension medium (0.9% NaCl, 0.1% Tween 80) and vigorously shaking for 1 min. The spore suspension containing 1 x 10^8 spores/ mL was used as the inoculum.

Selection and preparation of solid substrates

Several agro-industrial residues were obtained from the local market, Bangalore, Karnataka, India. Various agro-industrial residues such as bengal gram husk (BG), black gram husk (BH), coconut oil cake (COC), vegetable

waste (VW), green gram husk (GH), orange peel (OP), pineapple waste (PW), potato peel (PP), red gram husk (RG), rice bran (RB), rice straw (RS), saw dust (SD), sugarcane bagasse (SB) tea dust (TD), and wheat straw (WS) were evaluated for their potential as substrate in SSF for lovastatin production. All substrates were dried at 50 °C for 2 h.

Optimization of fermentation conditions for lovastatin production

Lovastatin production by endophytic strain *Aspergillus niger* was optimized under SSF. Various parameters analyzed included incubation temperature (22 – 35 ±2 °C), pH (3.5 – 8.5), incubation period (1 to 10 days), initial moisture content of the substrate (50 – 85 % v/w), particle size ranging from 0.4 - 1 mm, different bed depths (3, 7, 11 and 15 cm) and loss of organic matter were evaluated.

Extraction of lovastatin

At the end of SSF, the fermented material was dried at 50 °C for 24 h, powdered and 2 g of the powdered material was extracted with ethyl acetate (pH 3.0) in 250 mL Erlenmeyer's flasks. It was then incubated at 28 ±2 °C in rotary shaker at 200 rpm for 2 h. Then the mixture was centrifuged at 10,000 rpm for 10 min and supernatant was filtered through membrane filter with a pore size of 0.45µm. This supernatant is used for further analysis.

Quantitative analysis of lovastatin

To 1 mL of the supernatant, 1 mL of trifluroacetic acid (1%) was added and incubated for 10 min (Lactonization of hydroxyl acid form of lovastatin). From the above solution, 0.5 mL was taken and diluted 10 times with methanol and its absorbance was read at 238 nm by using UV-Visible Spectrophotometer [9].

RESULTS AND DISCUSSION

A detailed study of lovastatin by *A. niger* by solid state fermentation using several agro-industrial substrates of different particle sizes and under various conditions of pH, moisture content, incubation period and temperature was carried out to characterize the process.

Effect of substrates on lovastatin production

The critical factor in SSF (solid state fermentation) is the choice of a suitable substrate for the fermentation process [10]. To minimize the overall lovastatin production cost; lovastatin is produced using several cheap agro-residues. The production medium should be often simple, using agro-industrial by-products like wheat bran, rice bran or wheat straw as substrate [11]. Table 1 depicts that rice bran is the most suitable substrate which has induced the lovastatin production up to the highest levels (0.98 ±0.01 mg/g) followed by bengal gram husk, red gram husk and green gram husk respectively. Based on the screening study, rice bran was selected as suitable substrate for further optimization studies under SSF. It contains sufficient nutrients which can be used by microbes for their growth.

Table 1
Effect of various substrates on lovastatin production

AGRO INDUSTRIAL RESIDUES	LOVASTATIN (mg/g)	LOSS OF ORGANIC MATTER (%)
Bengal gram husk	0.86±0.02	46±0.16
Black gram husk	0.70±0.06	38±0.36
Coconut oil cake	0.13±0.07	14±0.23
Vegetable waste	0.35±0.02	36±0.18
Green gram husk	0.80±0.02	42±0.28
Orange peel	0.01±0.02	2.0±0.30
Pineapple waste	0.02±0.01	2.0±0.21
Potato peel	0.25±0.02	28±0.24
Red gram husk	0.85±0.02	45±0.28
Rice bran	0.98±0.01	59±0.24
Rice straw	0.55±0.02	22±0.25
Saw dust	0.68±0.02	38±0.15
Sugarcane bagasse	0.60±0.02	35±0.26
Tea dust	0.50±0.02	20±0.26
Wheat straw	0.52±0.01	20±0.21

Effect of temperature

Fermentation was carried out at various temperatures from 22-35 °C to study its effect on lovastatin production. Results presented in Fig. 1 indicated that maximum lovastatin production (1.0 mg/g dry substrate) was obtained when SSF was carried out at 28°C. Any temperature beyond the optimum range is found to have some adverse effect on the metabolic activities of the microorganisms. At temperatures lower or higher than that of optimum, less lovastatin production was observed. Decline in lovastatin production at higher temperatures might be due to denaturation of lovastatin or its inactivation at higher temperatures. Therefore, the subsequent experiments were conducted at an incubation temperature of 28 °C. With prolonged incubation, lovastatin activity decreased sharply suggesting that the end-point of fermentation should be carefully controlled because the synthesized lovastatin could be degraded by non-specific proteases secreted by the fungus [12]. These results are coinciding with those previously reported for lovastatin production by *Monascus ruber, Monascus purpureus* and *Aspergillus terreus* [13,14,15,16, 17].

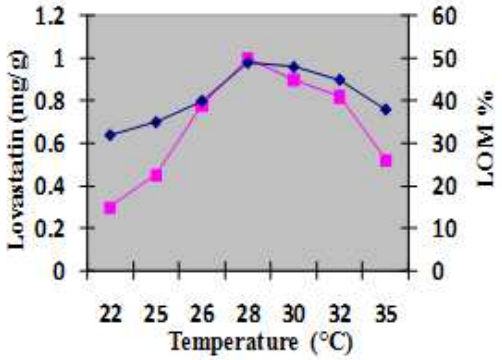

Figure 1
Effect of temperature on lovastatin production by endophytic fungal strain Aspergillus niger

Effect of pH

There exists a strong influence of initial pH of the medium on lovastatin production. To evaluate the effects of pH value in substrate on lovastatin production, the pH values were adjusted by the addition of HCl or NaOH to 3.5, 4.5, 5.5, 6.5, 7.5, and 8.5. The profound effect of initial pH of the fermentation on lovastatin production was shown in Fig 2. Maximum lovastatin yield (1.5 mg/g dry substrate) was recorded at pH 6.5.

A further increase in pH resulted in gradual decrease of lovastatin production due to the denaturation or inactivation of the microbial strain because pH strongly influences the transport of various components across the cell membrane which in turn supports the cell growth and product formation and most of the fungi are active in the pH range of 3.5-7.0 and also lower pH avoids the contamination by other microbes. The result was in accordance with that of Attalla *et al*[18]. who also investigated the maximum production of mevinolin (96.22 mg/l) at pH 6.5 by using *Aspergillus terreus.*

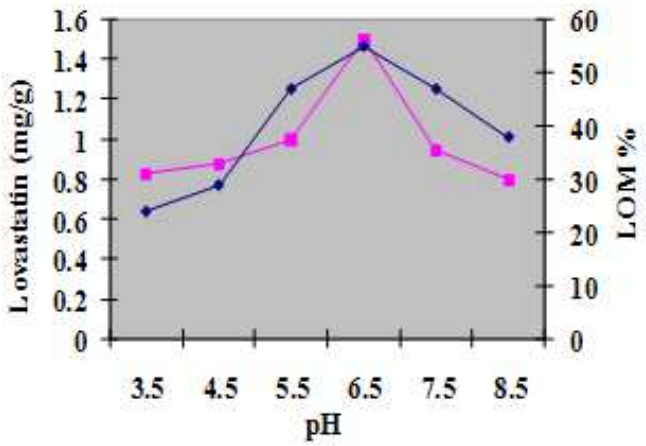

Figure 2
Effect of pH on lovastatin production by endophytic fungal strain Aspergillus niger

Effect of incubation period

To determine the optimum incubation period, fermentation flasks were incubated for different time duration (1 - 10 days). Lovastatin activity was analyzed at every 24 hrs time intervals. The production of lovastatin was observed from second day of incubation. The time course experiment revealed a steady increase in lovastatin production up to day eight, thereafter, the rate of production remained constant, indicating that 8 days incubation period is sufficient for maximum production of lovastatin (0.98 mg/g of dry substrate). It is clear from the plot (Fig .3) that initially as the LOM increased, while there was as lower increment in lovastatin content which means that most of the organic matter was utilized for fungal growth within that period. On the other hand, lovastatin production decreased with further extension of the incubation period to 11 days. Similar observations were reported by Siamak *et al* in case of *Aspergillus terreus*, they investigated the production of lovastatin at 7 days of incubation with a level of 55 mg lovastatin per liter of screening production medium. Previous reports indicated that incubation periods of 6-10 days were optimal for lovastatin production by various fungi [19,20,21,22].

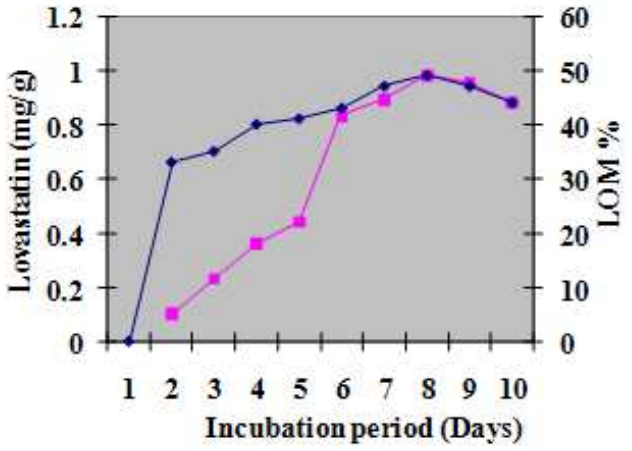

Figure 3
Effect of incubation period on lovastatin production by endophytic fungal strain Aspergillus niger

Effect of moisture content

The moisture content tested for maximum lovastatin production indicated enhanced lovastatin production with increase in the substrate moisture content up to 75 % beyond which it declined (Fig. 4). The highest production of lovastatin was obtained at a moisture level of 65 % and the maximum lovastatin activity was observed and it declined sharply at lower levels of moisture content. With moisture content below 50 % lovastatin production was decreased this may be due to metabolic heat produced during metabolic activity. However, when the moisture level was increased to 85 %, biomass activity as well as lovastatin decreased. This is presumably due to poor oxygen availability caused by excessive replacement of air by water in the void volume. Likewise, moisture level below optimum leads to reduced solubility of the nutrients of the solid substrate and lower degree of swelling of substrate [14]. The same 60% (v/w) moisture content was also observed for both *Aspergillus flavipes* and *Aspergillus terreus* under SSF[13, 23].

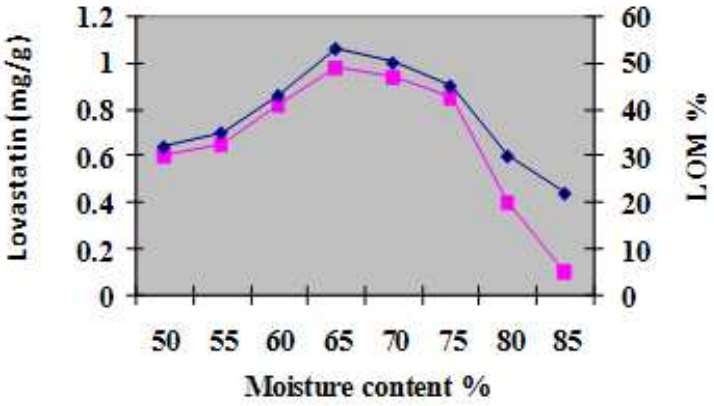

Figure 4
Effect of moisture content on lovastatin production by endophytic fungal strain Aspergillus niger

Influence of particle size

The results indicated that the substrate particle P3 (0.6 to 0.4 mm) was more suitable for lovastatin production (Fig. 5). Generally, smaller substrate particles will provide larger surface area for the attachment of microbes, which is favorable for mycelia growth and product accumulation. However, if the substrate size is too small, it may result in substrate agglomeration after heating in most cases, thus the reduction of inter-particle void space and the increase of oxygen mass transfer resistance are unfavorable for mycelia growth and lovastatin production. The results obtained are in accordance with several authors [13, 23].

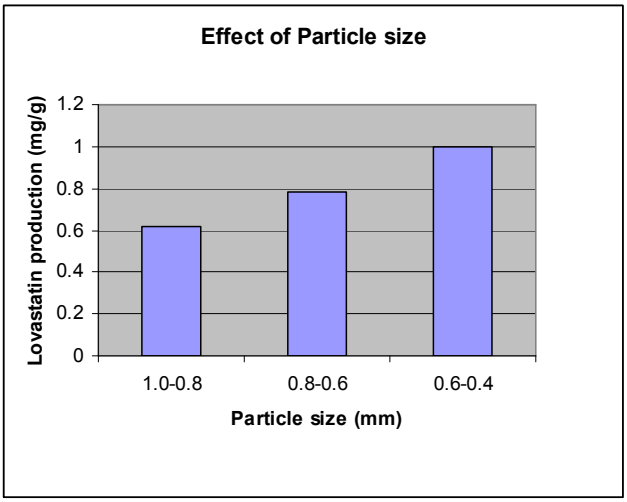

Figure 5
Effect of particle size on lovastatin production by endophytic fungal strain Aspergillus niger

Influence of bed depth

To confirm the role oxygen in lovastatin production, different bed depths of rice bran (3 cm, 7 cm, 11 cm and 15 cm) were studied (Table 2). In case of 3 cm bed depth, the growing fungi is more or less uniform throughout the bed yielding 1.0mg/g of dry solid and 0.80 mg/g of dry solid at top and bottom of the bed respectively with average lovastatin content of 0.91 mg/g of dry solid. As the bed depth increased from 3 cm to 15 cm, lovastatin content at the top and bottom of the bed as well as the average lovastatin content decreased drastically. This is presumably due to better molecular diffusion of air (O_2) from the stagnant gas phase of the head space through the interparticle voids into the biomass film on the surface of particles [24]. If the bed depth crossed the critical value (the depth below which the oxygen concentration falls to zero during the fermentation), there will be little or no growth in that region [25]. The critical bed depth is mainly a function of substrate particle size, substrate packing and vessel geometry. In this investigation, no growth observed at 15 cm bed depth and no traces of lovastatin was found.

Table 2
Effect of bed depth on lovastatin production by endophytic strain Aspergillus niger

Total bed depth cm)	Depth (cm)	Lovastatin (mg/g)	Average lovastatin content (mg/g)
3	0	1.0	0.91
	2	0.95	
	3	0.80	
7	0	0.99	0.75
	3	0.81	
	5	0.70	
	7	0.50	
11	0	0.95	0.57
	5	0.73	
	7	0.43	
	11	0.20	
15	0	0.80	0.47
	7	0.40	
	10	0.22	
	15	0.0	

CONCLUSION

The current investigation was mainly focused on the evaluation of the potentiality of endophytic fungi *Aspergillus niger* for utilization of agro-residual wastes as substrate for the production of lovastatin under solid state fermentation. With rice bran, the maximum yield of lovastatin (1.5 mg/g dry substrate) was achieved with the following optimized culture conditions on 8^{th} day of incubation at an optimum pH 6.5, temperature 28 °C, moisture content 65%, particle size of 0.6 to 0.4 mm and bed depth 3 cm. The yields obtained in the present study would have to be further increased for its industrial importance but it has proved the feasibility of solid state fermentation as a promising technique in exploiting cheaply available agro-residual wastes as substrates for the large-scale production of microbial metabolites of biotechnological importance ultimately leading to an effective solid waste management.

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