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Optimization of Process Parameters for Lovastatin Production from Red Gram Bran by Solid State Fermentation

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Abstract: ***Lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, was produced by solid state fermentation using protoplast fusant culture obtained by interspecific protoplast fusion between wild strains of Aspergillus terreus and Aspergillus flavus. A stable fusant strain was obtained after three times regeneration is used for lovastatin production. Different substrates such as black gram husk, coconut oil cake, corn flour waste, cotton waste, tea dust, groundnut shell, orange epicarp, pineapple epicarp, red gram bran, green gram bran, saw dust, banana peel, soybean meal, sugarcane bagasse and cauliflower leaf waste were studied to optimize the best substrate. The red gram bran showed the highest lovastatin yield of 0.91 mg/g of lovastatin. The physical parameters were optimized for maximum lovastatin production. Red gram bran of particle size 0.6-0.4 mm with the moisture content of 65% and pH 5.0 after 6 days of incubation with the strain yielded a maximum of 1.08 mg/g oflovastatin***

Keywords: Red gram bran, solid state fermentation, lovastatin, optimization, 3-hydroxy-3-methylglutaryl-coenzyme A

1. Introduction

Lovastatin, a fungal metabolite is a hypercholestroemic agent. This was the first natural statin from fungal origin to obtain approval from the US food and drug administration in August, 1987 (Manzoni and Rollini, 2002). This natural statin has been reported to be produced by Aspergillus *terreus* (Alberts et al., 1980), *Monascusruber*(Negishi et al., 1986), some *Penicillium*species (Endo et al., 1976) and *Trichoderma*species (Endo et al., 1986). The lipid lowering mechanism of this statin involves the reversible and competitive inhibition of HMG-CoAreductase enzyme, the rate limiting step in the cholesterol biosynthesis (Timothy et al., 1997). Lovastatin have shown great promise beyond their well-documented lipid lowering effects and they also suppresses a variety of leukemia cell lines and a wide array of solid tumor cells *invivo*, by inhibiting the synthesis of non-sterol isoprenoid compounds (Lewis et al., 2005). As a consequence of its importance, improving the production of lovastatin has become the focus of renewed research interest.

Lovastatin is an intracellular product and mostly accumulated in mycelia. In submerged fermentation, its yield is proportional to the amount of biomass, the high cell density increases the fermentation broth viscosity and the difficulty in stirring and oxygen mass transfer; an alternative strategy to produce lovastatin is by solid state fermentation (SSF) process. SSF compared to submerged fermentation is more simple, requires lower capital, has superior productivity, reduced energyrequirement, simpler fermentation media and absence of rigorous control of fermentation parameters, less water requirement, produces lower wastewater, easy control of contamination and requires low cost for downstream processing (Pandey, 1994; Babu and Satyanarayana,1995). SSF is a promising tool in biotechnology field for the production of microbial metabolites through inexpensivemeans and it is the most appropriate process for developing countries (Couto and Sanroman, 2006).Very few reports refer to lovastatin production in SSF Valera et al. (2005) with *A. flavipes*, Xu et al. (2005) with *M. rubber*, Wei et al. (2005) with *A. terreus* and solidliquid fermentation Chang et al. (2002) with *M. rubber*. The main objective of this study was to develop a potential fermentation process for the production of lovastatin employing SSF using agro-residues and also studying the effect of different parameters in an attempt to maximize lovastatin production.

2. Materials and Methods

2.1 Preparation of seed culture

The pure culture of protoplast fusant strain obtained by interspecific protoplast fusion between wild strains of *A. terreus* and *A. flavus* were maintained on potato dextrose agar slants, stored at 4°C and sub-cultured for every two weeks. For preparing a sporesuspension, to a well-sporulated slant, sterilized 0.1% Tween-80 solution was added. The surface was scratched with an inoculation needle and agitated thoroughly using cyclomixer to suspend the spores. The concentration of the spore-suspension was measured using a haemocytometer and adjusted to 1×10^8 spores /ml by diluting it suitably.

2.2 Solid state fermentation

Solid substrates were dried in an oven at 60°C, 10 g accurately weighed to Petri plates and was sterilized at 120°C for 30 min. After cooling, the solid substrates were inoculated with fungal spore suspension (10%, V/W). The final moisture content of the media was adjusted to 60% by

adding distilled water. Media was thoroughly mixed well with a stainless steel spatula and incubated at 30°C, in a humidity-controlled incubator for 6 days.

2.3 Lovastatin extraction and assay

Fermented material was dried at 40°C for 24 h and crushed to powder. The powdered material 2 g was extracted by 100 ml of methanol: water mixture (1:1, v/v) of pH 7.7 in 250 ml Erlenmeyer flask and keeping the flask at 30°C in rotary shaker at 200 rpm for 2h. After 2 h, the mixture was centrifuged at 10,000 rpm for 10 min and the supernatant was filtered through 0.45µm membrane filter. The filtrate was collected in vials and preserved at 4°C for further analysis. Lovastatin in the clear extract was estimated by high performance liquid chromatography (HPLC) (Shimadzu LC 8A) using a C18 column. A mixture of 0.02 M phosphate buffer (pH 7.7) and acetonitrile in the ratio of 65:35 (v/v) was used as mobile phase. The mobile phase flow rate was maintained at 1.0 ml/min and lovastatin was detected at 238 nm.

2.4 Effect of various substrates on lovastatin production

Agro-industrial residues were obtained from the local market, Coimbatore, Tamilnadu, India. The agro-industrial residues were crushed using a commercial waring type blender and used in the fermentation medium without any size differentiation. Various agro industrial residues such as, black gram husk, coconut oil cake, corn flour waste, cotton waste, tea dust, groundnut shell, orange epicarp, pineapple epicarp, red gram bran, green gram bran, saw dust, banana peel, soybean meal, sugarcane bagasse and cauliflower leaf waste were evaluated for their potential as substrate in SSF for lovastatin production. All substrates were dried at 60°C for 1 h. The best solid substrate achieved by this step was fixed for subsequent experiments.

2.5 Effect of incubation period

Different incubation periods (1 to 8 days) were employed to study their effect on lovastatin production. The fermentation was carried out at 30°C keeping all other conditions at their optimum level. The optimum incubation period achieved by this step was fixed for subsequent experiments.

2.6 Effect of initial moisture

In order to understand the effect of water availability, substrate swelling and the oxygen diffusion, the fermentation was carried out with different moisture levels (50, 55, 60, 65, 70, 75 and 80%) which were adjusted with distilled water. The moisture content suitable for maximum production was followed for subsequent setups.

2.7 Effect of the initial culture pH

To evaluate the effect of pH on lovastatin production, the pH values were adjusted to 4.0, 5.0 6.0, 7.0, 8.0 and 9.0 with 1 N HCl or 1 N NaOH. Other parameters were kept at their optimum levels.

2.8 Effect of particle size

The effect of particle size of the solid substrate on the production of lovastatin was studied by growing the fungus in three different milled stocks (S1, S2 and S3). S1 consisted of particles that were larger than 0.8 mm with a maximum particle size of 1.0 mm. S2 composed of particles smaller than 0.8 mm with minimum size of 0.6 mm and S3 particles ranges from 0.6 mm to 0.4 mm. The optimum particle size of the substrate achieved by this step was fixed for subsequent experiments.

2.9 Effect of bed depth of the substrate

To find out the effect of bed depth of the substrate, experiments were performed in 5 litre bottles (20 cm OD) with four different bed depths (4, 8,12,16 cm) of red gram bran. Thoroughly dried red gram bran of particle size S3 was transferred in different bottles tomaintain a particular bed depth in each bottle. Optimal levels of the other derived factors were used.

2.10 Loss of organic matter

There is no direct method for biomass estimation in SSF. Loss of organic matter (LOM) was used to express biomass and metabolic activity in an indirect way (Kumar et al., 2003). Fermented substrate was withdrawn and weighed (W1), and then dried at 60°C until constant weight (W2). LOM was calculated as the weight difference and expressed as a percentage of the initial dry weight of the samples of the fermented substrate and it can be calculated as follows:

$$LOM = (W1-W2)/W1\times100\%$$

Where, W1 is the initial dry weight of solid material before fermentation and W2 is the final dry weight of solid material after fermentation.

3. Results and Discussion

3.1 Selection of substrate

Filamentous fungi are capable of penetrating into the hardest of solid substrates, aided by the presence of turgor pressure at the tip of the mycelium (Ramachandran et al., 2004). Various agro-products including black gram husk, coconut oil cake, corn flour waste, cotton waste, tea dust, groundnut shell, orangeepicarp, pineapple epicarp, red gram bran, green gram bran, saw dust, banana peel, soybean meal, sugarcane bagasse and cauliflower leaf waste were evaluated to select the best supporter of lovastatin production by a stable protoplast fusant strain. For all media, the mycelia were harvested at the 6th day of fermentation. It was remarked that the most abundant visible growth was obtained using red gram bran as substrate, probably due to its high content of carbonaceous nutrients. Conversely, non-visible fungal growth was observed when sugarcane bagasse and cauliflower leaf waste were employed. Red gram bran served as the best substrate for higher lovastatin production substrate followed by green gram bran. The order of substrate suitability was red gram bran> green gram bran > black gram peel > banana peel >corn flour waste > tea dust > orange epicarp> pine apple waste > cotton waste > coconut oil cake > saw dust >sugarcane bagasse >

cauliflower leaf waste >soya bean meal (Table 1). Wheat bran was used quite frequently by other researchers to produce lovastatin (Valera et al., 2005; Wei et al., 2005). It is the first time that red gram bran was used as solid substrate to support lovastatin production. The selection of an ideal agro-biotech waste for lovastatin production in a SSF process depends upon several factors, mainly related with cost and availability of the substrate material. Based on the above study, it was evident that red gram bran acts as a best substrate for the fusant fungal strains. The universal suitability of redgram bran may be due to insolubility in water, nature of cellulose and hemicellulose. It contains sufficient nutrients and is able to remain loose even when water is absorbed on to the substrate particles in moist conditions, thereby providing good aeration and large surface area, which can be used by microbes for growth and metabolic activity.

Table 1: Effect of different substrates in lovastatin production

Substrate	Lovastatin content (mg/g of dry solid)		LOM (%)	
	Sample 1	Sample 2	Sample 1	Sample 2
Cotton waste	0.32	0.35	29.71	30.92
Black gram peel	0.69	0.71	41.37	40.87
Com flour waste	0.52	0.59	33.04	34.02
Red gram bran	0.91	0.87	43.26	43.65
Banana peel	0.65	0.68	22.35	23.12
Ground nut shell	0.21	0.28	30.01	30.25
Orange epicarp	0.45	0.51	27.05	26.97
Saw dust	0.22	0.25	20.84	21.48
Cauliflower leaf waste	0.13	0.15	10.66	9.05
Sugarcane bagasse	0.21	0.23	14.73	14.82
Soybean meal	0.09	0.18	6.7	8.03
Pineapple epicarp	0.38	0.35	21.81	20.04
Tea dust	0.51	0.53	32.01	32.69
Coconut oil cake	0.26	0.24	11.28	11.31
Green gram bran	0.77	0.79	41.01	41.12

3.2 Role of incubation time

The time course experiment revealed a steady increase in lovastatin production up to day 6, thereafter, the rate of production remained constant, indicating a day 6incubation period as sufficient for maximum production of lovastatin (0.89 mg/g of dry substrate). The production of lovastatin was observed even after first day of incubation. It is clear from the plot (Figure 1) that initially as the LOM increases, there is a slower increment in lovastatin content which means that most of the organic matter is utilized for fungal growth within this period. After 3 days, lovastatin content increases rapidly with a very slow raise in LOM and attains maximum value after 6 days of incubation. This trend supports the fact that stationary phase is the most suitable phase for lovastatin production which is a fungal secondary metabolite.

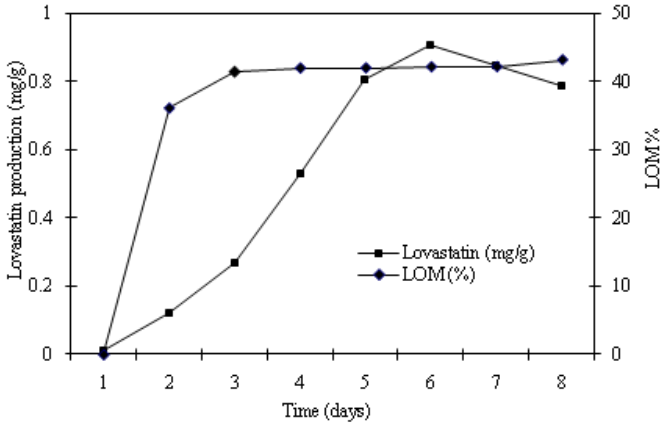

Figure 1: Time course of lovastatin production and LOM

3.3 Influence of pH

An important factor that affects the performance of red gram bran fermentation is the initial pH of the substrate. The purpose of this experiment was to determine the optimum initial pH of red gram bran that would result in the highest lovastatin yield. As shown in Figure 2, the lovastatin yield increased with the increase in initial pH from 3-5. As pH was increased further from 5-9, there was a steady decrease in lovastatin content as well as in LOM. Results clearly indicate that pH 5 (0.87 mg/g) is optimal for lovastatin production.

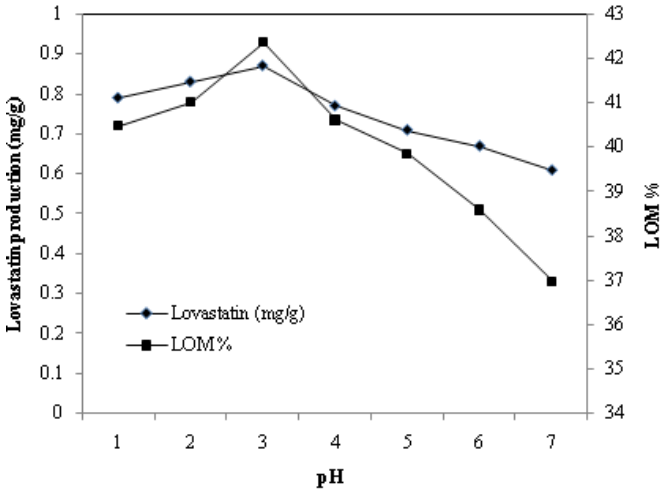

Figure 2: The effect of pH on the lovastatin production

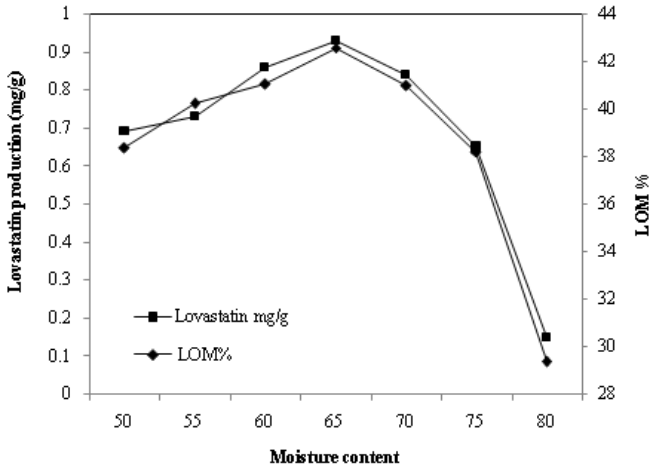

Figure 3: The effect of moisture content on the lovastatin production

3.4 Influence of moisture content

Among the several factors that are important for microbial growth and lovastatin production under SSF using a particular substrate, moisture level/water activity is one of the most critical factor in SSF which often determines the success of a process (Virupakshi et al., 2005; Nigam and Singh, 1994), because SSF processes are different from submerged fermentation culturing, microbial growth and product formation occurs at or near the surface of the solid substrate particle having low moisture contents (Pandey et al., 2000). In this investigation, different initial moisture content of the SSF medium was established by altering the volume of moistening solution added to the solid substrate. Figure 3 indicates that maximum lovastatin production was obtained with 65% moisture content (0.93 mg/g). Reduced lovastatin production resulted from further increase in moisture content. With 50% moisture content, the available oxygen is sufficient but the water content is not enough to support good metabolic activity and removal of metabolic heat. This may account for lower lovastatin content and LOM in this case. As the moisture level was increased from 50% to 70%, lovastatin production increased. This may be because of the adequate amount of oxygen and water present within the substrate bed to support good fungal growth and also to remove metabolic heat. However, when the moisture level was increased to 80%, biomass activity as well as lovastatin content decreased. This is presumably due topoor oxygen availability caused by excessive replacement of air by water in the void volume. For this investigation, 65% moisture level was found ideal for lovastatin production.

3.5 Influence of particle size

In SSF process, the availability of surface area plays a vital role for microbial attachment, mass transfer of various nutrients, subsequent growth of microbial strain and product production. It affects the surface area to volume ratio of the particle, which is initially accessible to the microorganism (Krishna, 1999). It is known that the void age increases and surface area decreases with increasing particle size and vice versa. Experiments were carried out with three different particle sizes. In case of particles S1 (1-0.8 mm), void age is higher because the particles are larger, the amount of trapped air is also higher but the substrate availability is lesser because of lower surface area. This may account for lower lovastatin content and LOM. The maximum yield of lovastatin (1.01mg/g of dry solid) was obtained using particles S3 (0.6-0.4 mm), because of higher substrate availability and surface area for particle S3 (Figure 4). This may be the reason that the highest metabolic activity 44.25% LOM (result not shown). For the particles S2 (0.8-0.6 mm)result lie in between those of particles S1 and S3 respectively.

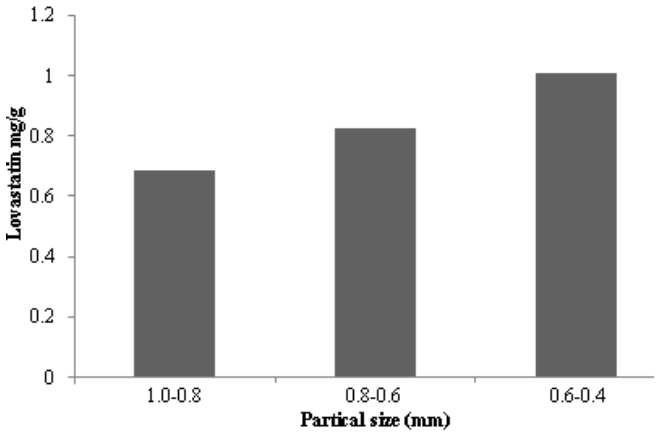

Figure 4: The effect of particle size on the lovastatin production.

3.6 Influence of bed depth

To confirm the role of oxygen in lovastatin production experiments were performed in closed 5 litre bottles (20cm OD) without supplying additional air. Different bed depth of

red gram (4 cm, 8 cm, 12 cm and 16 cm) were studied (Table 2). In case of 4 cm bed depth, the growing fungi is more or less uniform throughout the bed yielding1.08 mg/g of dry solid and 0.81 mg/g of dry solid at the top and bottom of the bed, respectively with average lovastatin content of 0.98 mg/g of dry solid. As the bed epth increased from 4-16 cm, lovastatin content at the top and bottom of the bed as well as the average lovastatin content decreased drastically. The results show that the lesser the bed depth of substrate, the higher lovastatin production. This is presumably due to better molecular diffusion of air (O2) from the stagnant gas phase of the headspace through the inter particle voids into the biomass film on the surface of particles (Rajagopalan et al., 1994). If the bed depth crosses the critical value (the depth below which the oxygen concentration falls to zero during the fermentation), there will be a little or no growth in that region (Raghavarao etal., 2003). The critical bed depth is mainly a function of substrate particle size, substrate packing and vessel geometry. In this investigation, very less growth was observed at the bed depth of 10 cm and no growth was observed at 16 cm bed depth with 0.00 mg/g of lovastatin.

In this work, red gram bran was found to be a suitable substrate for lovastatin production by protoplast fusion strain in the SSF process. The optimum productivity of lovastatin (1.03 mg/g) was achieved by employing red gram bran with optimized process parameters such as incubation period of 6 days, moisture content 65%, mesh size of 0.6 mm-0.4 mm and pH 5. Under the optimal process parameters, the maximum lovastatin yield of 2.9mg/g dry substrate was obtained after 6 days of fermentation. These results indicate that optimized culture conditions can be used for industrial production of lovastatin to obtain high yields.

Table 2: Effect of bed depth on lovastatin production.

Bottle number	*Total bed depth(cm)*	*Depth (cm)*	*Lovastatin content* (mg /g of dry solid)	*Average lovastatin content* (mg /g of dry solid)
1	4	0	1.08	
		2	0.93	0.98
		4	0.81	
2	8	0	1.01	
		3	0.89	0.8
		6	0.73	
		8	0.48	
3	12	0	0.91	
		4	0.83	0.75
		8	0.74	
		12	0.28	
4	16	0	0.83	
		5	0.68	0.51
		10	0.53	
		16	0	

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