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**PRODUCTION OF A CHOLESTEROL LOWERING DRUG -  
LOVASTATIN BY *ASPERGILLUS TERREUS* USING  
POLYURETHANE FOAM AS AN INERT SUPPORT MATERIAL IN  
SOLID STATE FERMENTATION**

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**ABSTRACT**

Solid state fermentation (SSF) on inert supports is very convenient for basic research and may also have potential as a high-production commercial system. Polyurethane foam (PUF) was used as inert support for lovastatin production by two isolates of *Aspergillus terreus* (18 and 76). Results indicated that 7 foam pieces (1cm× 1cm× 1cm), 10ml of 1X media containing lactose (40g/L) supported highest lovastatin yield by both the isolates by 5<sup>th</sup> day of incubation. Lovastatin yield of 7.27 mg/g dry biomass and 10.82mg/g of dry biomass by *A.terreus* 18 and 76 was observed on PUF immobilization which was 2.5 and 4 folds higher than the yields obtained by these two isolates, respectively, in free cell conditions on SSF cultivation with wheat bran (1g). Additionally, repeated batch biotransformation with immobilized fungal cells on flexible PUF could be successfully maintained for 2 cycles of 1 day interval each after the first cycle of 5 days. The highest yield of lovastatin reached to 7.27 mg/gds and 10.82 mg/gds by *A.terreus* isolates 18 and 76, respectively, in the first batch and supported comparable yields in the 2<sup>nd</sup> cycle with decreased fermentation time (1 day interval). PUF supported with lactose media supported higher lovastatin yield (~1.6 fold) by both isolates as compared to free cells indicating the importance of PUF support in SSF for lovastatin production.

**Key words:** Secondary metabolite, Lovastatin, Polyurethane foam, wheat bran, repeated batch

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## INTRODUCTION

Lovastatin (Mevinolin, Monocolin K, and Mevacor<sup>TM</sup>) is potent drug for lowering blood cholesterol. Lovastatin act as a competitive inhibitor to the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA) which catalyzes the rate limiting step of cholesterol biosynthesis [1]. Because of the hypocholesterolemic and hypolipaeamic properties of lovastatin, search for efficient producers and perfection of improved production process are of interest [2]. Several fungal genera including *Aspergillus*, *Penicillium*, *Monascus*, *Paecilomyces*, *Trichoderma*, *Scopariopsis*, *Doratomyces*, *Phoma*, *Phythium*, *Gymnoascus*, *Hypomyces* and *Pleurotus* have been reported to be able to produce lovastatin [3].

Fermentation of immobilized microbial cells has recently gained much attention among many biotechnological approaches, because of its advantage over conventional free cell systems with respect to retention of high cell density, operational stability, higher efficiency of catalysis, higher volumetric productivity and lower shear stress [4]. The adsorption in porous material such as polyurethane foam (PUF) is a very simple immobilization used in liquid fermentation [5]. Many examples of microbial cell immobilization on PUF have been reported in literature, such as bacteria [6], microalgae [7], basidiomycetes [8],

ascomycetes [9] and Cyanobacteria [10]. It is well known that designing proper culture conditions is a prerequisite in the production of metabolites [11].

Earlier studies for the production of lovastatin by SSF has been made on various types of oil cakes, agrowastes and fruit waste. Very few studies has been done on the production of lovastatin on polyurethane foam. This method has certain advantages, such as being a relatively clean system, maintaining a constant physical structure throughout the process, and offering the possibility of using liquid medium with any desired composition. Thus, SSF on inert supports is very convenient for basic research and may also have potential as a high-production commercial system [12].

The main aim of this experiment was to find out the optimum conditions for the production of lovastatin on polyurethane foam, to observe the effect of large surface area on production as compared to traditionally available raw material like wheat bran for the production of lovastatin.

## MATERIAL AND METHODS

### Microorganism and inoculum preparation

Two different species of *Aspergillus terreus* were isolated from soil samples.

They were maintained on Potato dextrose agar plates. Spore suspension was prepared using 10 ml of sterile distilled water along with 0.1% v/v) tween 80 in a pure culture plate. The spore surface was scrapped with an inoculating loop to suspend the spores in the solution and the obtained spore suspension was used as the inoculum for the fermentation process.

### **Immobilization on polyurethane foam**

The polyurethane foam material used for the immobilization studies had a porosity of 100-500  $\mu\text{m}$ . It was cut into  $1\text{cm}^2$  pieces [13] and washed with distilled water. Different number of foam pieces ranging from 7 to 28 were submerged in a 250 ml Erlenmeyer flask containing 50 ml of the fermentation medium consisting of lactose (10g/L); and then sterilized at  $121^\circ\text{C}$  for 15 min [13]. This was compared with a PUF free lactose medium.

### **Fermentation processes**

#### *Batch fermentation with immobilized and free cells*

The batch experiments were performed in 250 ml Erlenmeyer flasks each containing 50 ml of fermentation medium. The free-cell fermentation was carried out by adding the spore suspensions of *A.terreus* 18 and *A.terreus* 76 separately to the medium without supporting matrix. Parallel experiments were conducted with immobilized cells by inoculating the flask

containing polyurethane foam pieces with the spore suspension [13]. The flasks were incubated at  $30^\circ\text{C}$  in a shaker incubator maintained at 200 rev/min. After the stipulated fermentation time the samples were detected for lovastatin yields.

Different parameters like number of foam pieces (7,14, 21 and 28); media volume (10ml, 20ml, 30ml, 40ml and 50ml); media strength (0.5X, 1X, 2X and 4X) and fermentation period (1- 5 days) were optimized under immobilized conditions by changing the parameters one at a time.

### **Lovastatin extraction and estimation**

After fermentation, polyurethane was pressed with autoclaved glass rod to get culture filtrate containing lovastatin. pH of culture filtrate was adjusted to 2 and equal amount of ethyl acetate was added, kept in a rotary shaker for 2 h. After 2 h, broth was separated from organic phase and organic phase (Ethyl acetate) was allowed to dry. Dried residues were dissolved in 1ml ethanol and estimation of lovastatin was carried out at 510 nm using colorimeter [14].

### **Repeated Fermentation with Immobilized Cubes**

One of the advantages of using immobilized biocatalysts is that they can be used repeatedly and continuously [15]. Therefore, the reusability of cells immobilized on polyurethane foam was

examined. In case of repeated batch studies, the fermentation medium was aseptically decanted from each flask on 5<sup>th</sup> day after the first cycle and fresh medium was then added. The process was continued for the next cycle. The second cycle was harvested at one day interval upto 10 days and lovastatin yield after every harvest was determined.

### Statistical analysis

All the experiments were done twice with 3 replicates. Individual culture flasks were considered as experimental units. Data was analysed by One Way ANOVA and all multiple comparisons among means were performed using Duncan's new multiple range test ( $\alpha = 0.05$ ).

## RESULTS AND DISCUSSION

Polyurethane foam has been used as an artificial inert support. Soyabean meal media containing lactose as a carbon source was used to carry out the process of fermentation. Polyurethane foam has number of pores on the surface which increase the surface area for fungal mycelia to grow.

Solid state fermentation at pH 6, 30°C incubation temperature, 1g wheat bran, 70% initial moisture content, and 5 days fermentation time, without addition of any other nutrient sources yielded maximum production of lovastatin of 2.58 mg/g dry

substrate and 2.5 mg/g dry substrate by *A. terreus* 18 and 76, respectively [16].

### Number of foam pieces

Number of foam pieces significantly influence both biomass and lovastatin production by the two *A. terreus* isolates 18 and 76 ( $p < 0.05$ ). Biomass of *A. terreus* 18 and 76 increased with increase in foam pieces from 7 to 28. However this was accompanied by a decrease in the lovastatin yield (Fig. 1). 7 pieces of PUF supported the maximum lovastatin production in case of both isolates-(isolate 18 and 76). Maximum yield obtained in case of isolate 18 was 1.33mg/g of dry biomass and in case of isolate 76 maximum yield obtained was 1.11mg/g of dry biomass.

It was observed that with the increase in the number of foam pieces growth of mycelia also increased but very soon fungal cells enter into death phase from stationary phase. Lovastatin gets produced in the stationary phase of the life cycle of fungal cells [1]. With the increase in number of PUF pieces length of stationary phase got reduced thus leading to a decrease in lovastatin yield.

### Media volume

Of the different volumes of media tested for fermentation under immobilized condition, 10 ml of the media added to PUF mimicked the condition of solid state fermentation. Different amount of media

supported different yield of lovastatin. It was observed that as increase in media amount led to the increase in biomass. Results showed that 50ml of media with 7ps of PUF supported the minimum production of lovastatin. Maximum amount of lovastatin was supported by 10 ml of media (Fig. 2). Yield of lovastatin obtained by isolate 18 was (6.14 mg/g of dry biomass) and isolate 76 supported (7.63 mg/g of dry biomass) of lovastatin. Increase in media amount increases the length of log phase delaying the onset of stationary phase, thereby decreasing lovastatin production.

#### **Media strength**

Media of different strength was used for the production of lovastatin. Strength of media was varied from 0.5X to 4X. It was observed that as the strength of media increased growth of biomass also increased with concomitant decrease in lovastatin yield (Fig.3). 0.5 X media did not support growth. Maximum lovastatin yield was supported by IX strength of media. Similar observations were reported where they have investigated the effect of medium concentration on the growth of biomass and found that biomass concentration increased with the increase in medium concentration [17] which affected lovastatin yield.

#### **Repeated batch fermentation**

Using PUF as an inert support production of lovastatin was carried out for 2 cycles at different time periods of the second cycle to check the decrease in duration required for lovastatin production once the biomass is ready in immobilized state after the first cycle. In the first cycle maximum amount of lovastatin was obtained on 5<sup>th</sup> day in case of both isolates (7.27 mg/gds by isolate 18 and 10.82 mg/gds by isolate 76) after which comparable yield was obtained within one day of cycle 2 (6.52mg/gds by isolate 18 and 9.46mg.gds by isolate 76) upto 5 days though the yield gradually decreased.

The mouldy substrates had been tried to be used in solid state fermentation for multiple cycles of fermentation. The disadvantages of this method usually lied in: (1) If the substrates were washed before being reused to get the maximal harvest of the products, the damage to the cells would occur which would bring negative effects to following fermentation; (2) If the substrates were reused directly without any washing, the yields of the product would be reduced as some of the substrates was recycled for next batch of fermentation. In the fermentation process with PUF, the substrates will be adsorbed to the PUF and the mycelia of the fungi will grow in the pores of the PUF. In our study, when the

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maximal yield of lovastatin was obtained at the end of 5 days, the PUF adsorbed with cells was separated from the solid substrate and tried to be reused in the fresh substrates for semi-continuous fermentation (Fig. 4).

In this research, repeated-batch fermentation was used in the conversion process of sugars to ethanol because this process has several advantages compared to conventional batch fermentation such as no new inoculum is required for each batch and long-term productivity. In addition, no time is wasted for cleaning and resterilization, and the operational control is easier than that of a continuous mode. In the repeated-batch process using free cells, the portion of the fermented broth is withdrawn at time intervals, and the residual part of the broth is used as an inoculum for the next batch. However, this method causes a reduction in microbial cell concentration, resulting in lower ethanol production in the subsequent batches. To avoid this phenomenon, the repeated-batch process using immobilized yeast cells is proposed. The use of immobilization systems can minimize the production costs,

because this system offers several advantages over the free cell fermentation operation *i.e.*, higher cell concentration, higher fermentation rate, easier cell recycle and lesser product inhibition [18].

In a batch process, a typical fermentation cycle comprises of a growth phase followed by a production phase and finally a production stop phase [19], and the recycling of microorganism might be beneficial for reducing fermentation time and improving productivity. Similar results were obtained in this study.

PUF supported with lactose media supported higher lovastatin yield (~1.6 fold) by both isolates as compared to free cells indicating the importance of PUF support in SSF for lovastatin production (Fig.5).

The results in the study showed that fungus immobilized in the PUF was successively reused to produce lovastatin after feeding fresh substrates, however further investigation of the process is still needed as the maximum lovastatin production in the following batches is less than that in the first batch fermentation.

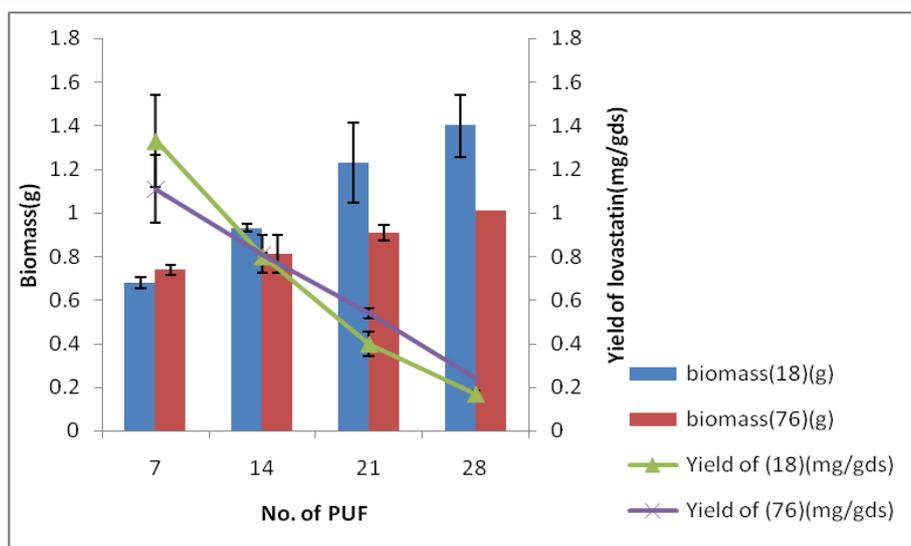


Fig.1. Effect of different number of PUF pieces on fungal biomass and lovastatin yield of *A.terreus* 18 and 76

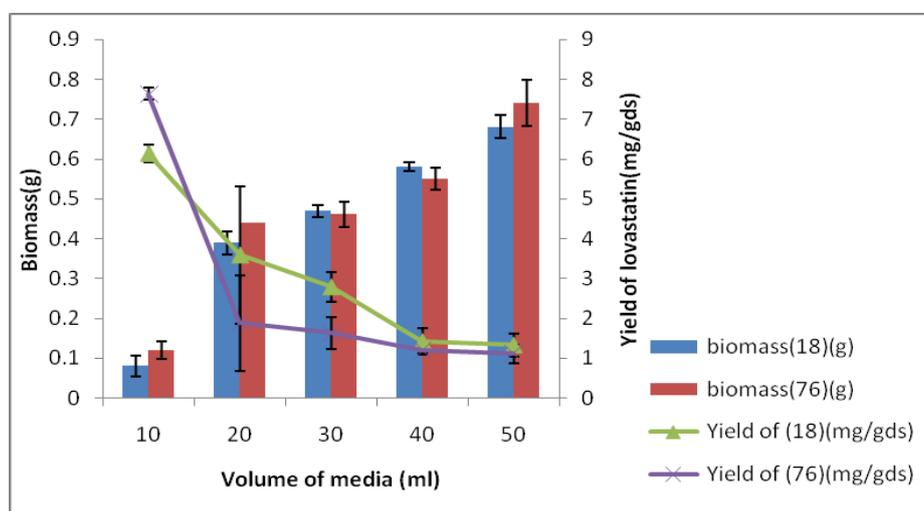


Fig.2. Effect of media volume with PUF on biomass and lovastatin yield of *A.terreus* 18 and 76

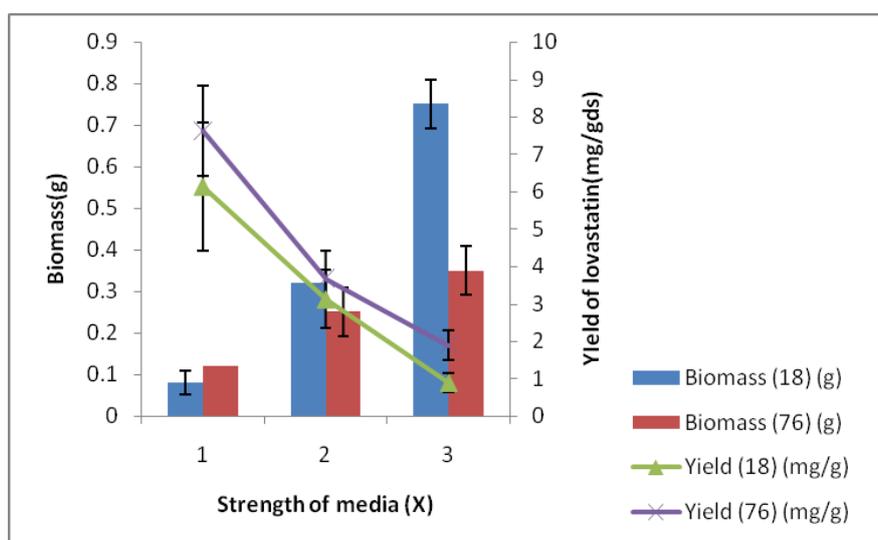


Fig.3. Effect of media strength on biomass and lovastatin of *A.terreus* 18 and 76.

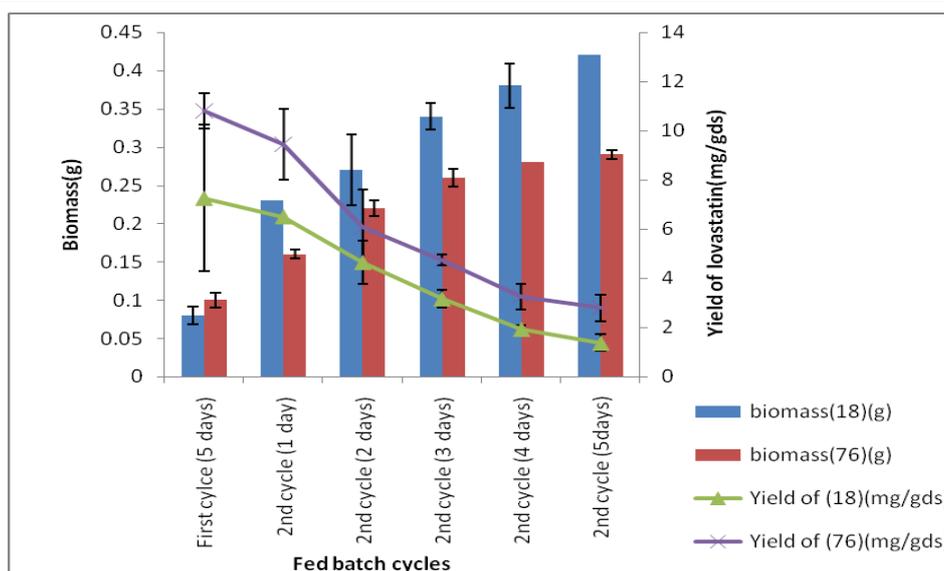


Fig.4. Repeated batch fermentation of *A.terreus* 18 and 76 using PUF-lactose media.

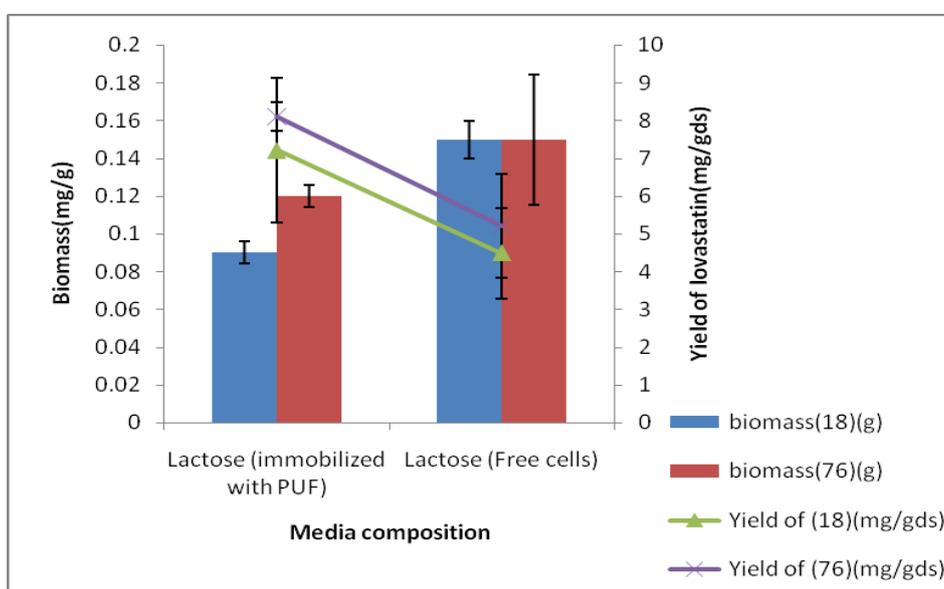


Fig.5. Effect of PUF support on biomass and lovastatin of *A.terreus* 18 and 76 in lactose media.

## CONCLUSION

This study shows appreciable increase in yield of lovastatin by immobilised cells as compared to solid state fermentation using wheat bran. It also proved that Poly urethane foam (PUF) can act as an artificial growth supporting material for semi-continuous fermentation reducing the cycle

interval for successive cycles of production of lovastatin.

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