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Enhanced Continuous Production of Lovastatin Using Pellets and Siran Supported Growth of *Aspergillus Terreus* in an Airlift Reactor

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Abstract Lovastatin, a hypocholesterolemic agent, is a secondary metabolite produced by filamentous microorganism *Aspergillus terreus* in submerged batch cultivation. Lovastatin production by pellets and immobilized siran cells was investigated in an airlift reactor. The process was carried out by submerged cultivation in continuous mode with the objective of increasing productivity using pellet and siran supported growth of *A. terreus*. The continuous mode of fermentation improves the rate of lovastatin production. The effect of dilution rate and aeration rate were studied in continuous culture. The optimum dilution rate for pellet was 0.02 h^{-1} and for siran carrier was 0.025 h^{-1}. Lovastatin productivity using immobilized siran carrier (0.0255 g/L/h) was found to be greater than pellets (0.022 g/L/h). The productivity by both modes of fermentation was found higher than that of batch process which suggests that continuous cultivation is a promising strategy for lovastatin production.
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Keywords: airlift reactor, Aspergillus terreus*, continuous fermentation, lovastatin, pellets, siran*

INTRODUCTION

Lovastatin is a secondary metabolite that acts as a hypocholesterolemic agent. It is produced by batch fermentation process in a complex media [1,2] using *Aspergillus terreus.* The metabolite production occur during the second phase of growth of microorganism, the idiophase (unbalanced growth phase) which follows the trophophase. In submerged culture, a large number of factors contribute to the development of morphological form which affects the fermentation process. Lot of efforts are being put globally to enhance the yield of lovastatin production at industrial level, which include better strains and process strategies improvement. Among the fermentation strategies adopted to improve the productivity, the whole cell immobilization technology appears to be attractive for fermentation [3]. Immobilized whole cells of microbes offers several advantages, they can work as effective biocatalyst for repeated batch and continuous production. The adsorption technique involves the utilization of surface attachment characteristics of microbes to the support.

Study was performed in an airlift reactor (ALR) that has advantage to prevent the mechanical damage to fungal pellets. The development of an improved strategy and proper control of specific growth rate for lovastatin production is required. As an alternative to batch culture, a fed batch strategy has been attempted by Kumar *et al.* [4]. Novak *et al.* observed that in fed batch fermentations synthesis of lovastatin could be prolonged compared to batch fermentation, but productivity was reduced [5]. From commercial viewpoint, continuous process can provide several advantages in comparison with batch conditions [6]. Continuous operation is more efficient than batch operation in terms of processing time since dead time; due to charge and discharge operation, bioreactor preparation and sterilization is avoided. In batch culture cells are in a constant changing environment, whereas in continuous run the cells are in better adapted stage. Earlier studies suggest the requirement of certain level of specific growth rate of organism for its maximum biosynthetic activity. Control on biomass in cultivation with sufficient supply of dissolved oxygen by maintaining aeration enables the maximization of production

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of secondary metabolites.

Keeping in view all these, we performed an experiment involving continuous process of lovastatin production that includes initial batch phase up to 72 h for the formation of biomass for lovastatin production and then proceeded with continuous supply of the medium at different dilution rate in ALR. To the best of our knowledge there has been no report about continuous lovastatin production by immobilized cell in an ALR.

The purpose of the present work was to investigate an alternate strategy to improve lovastatin production by *A. terreus* during submerged growth and continuously in an ALR using pellets and immobilized siran carriers. Continuous cultivation was performed to determine the optimum value of dilution rate and aeration rate for lovastatin production. The efficiency of the resultant process was compared with that of conventional batch fermentation.

MATERIALS AND METHODS

Organism and Fermentation Conditions

The production of lovastatin was carried out using *A. terreus* (NRRL 255). The strain was obtained from Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois, USA. It was maintained on PDA incubated at 28°C for 4~5 days and stored under refrigeration at 5~10°C.

Growth medium [7] was prepared using Glucose (45 g/L), KH_2PO_4 (5 g/L), K_2HPO_4 (5 g/L), Monohydrate sodium glutamate (12.5 g/L), and H_3BO_3 (11 mg/L). The trace element solution contains: $FeSO_4 \cdot 7H_2O$ (0.2 g/L), $MnSO_4 \cdot 4H_2O$ (0.1 g/L), $ZnSO_4 \cdot 7H_2O$ (0.2 g/L), $MgSO_4 \cdot 7H_2O$ (0.1 g/L), $CaCl_2 \cdot 2H_2O$ (20 mg/L), $CuCl_2 \cdot 2H_2O$ (5 mg/L), and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (5 mg/L). The fermentation was carried out at 28°C for 3~4 days on a rotary shaker at 180 rpm.

Production medium was prepared with Glucose (40 g/L), Milk powder (15 g/L), Soybean meal (5.5 g/L), Malt extract (0.5 g/L), Sodium acetate (1.0 g/L), Peptone (1.0 g/L), NaCl (0.2 g/L), $CaCO_3$ (1.5 g/L), KH_2PO_4 (0.05 g/L), $MgSO_4 \cdot 7H_2O$ (0.05 g/L), and Antifoam agent. The pH was adjusted to 5.8~6.0. For the optimization experiments the production medium containing flasks were incubated on a rotary shaker at 250 rpm and 28°C for 10 days.

Siran Supported Growth

Siran beads, obtained from Bioengineering AG, Switzerland, were shifted by sieve fractionation and particle sizes ranging from 600~1,000 μm were pooled for fermentation in ALR. These beads were washed thoroughly with water. Two hundred milliliter of 5% nitric acid was placed on a 100 g siran carrier and degassed under vacuum. Beads were then boiled in nitric acid for 12 h with running distilled water. The carriers were dried in an oven at 150°C. One hundred gram siran beads were autoclaved in dry state and 100 mL of spore suspension was added to the beads. One hundred milliliter of sterile water was also added.

Experimental Set-up

A 5.0 liter internal loop ALR was indigenously designed to study the highly aerobic lovastatin production process. The basic design of the lab scale ALR was determined from the earlier experiments [3]. Borosilicate glass was used for the construction of ALR. The dimensions were restricted to ease the sterilization process in laboratory autoclave. A constant air flow rate was maintained through an air filter and the flow rate was measured with a rotameter. All the ports of ALR were aseptically sealed after inoculation. Sterile air was sparged cocurrently through a single sparger and air flow rate was maintained.

Continuous Operation

The above batch operation was switched over to continuous operation mode by constantly adding the production medium. The overflow was collected constantly using a peristaltic pump. The feed dilution rates were varied from 0.01 to 0.05 h^{-1}. The fermentation was continued with optimum dilution rate. The reactor was fitted with a two channel peristaltic pump which provided both substrate feeding and exhausted effluent withdrawal. The feed was continuously supplied at the bottom. Feed rates were varied and the output collected at the top. The foaming was controlled by using silicone oil and the different parameters for continuous cultivation were observed.

Analytical Methods

Batch fermentation runs were conducted for 10 days of growth and lovastatin production. Various samples were collected every 24 h intermittently. Every run was done in triplicate. Samples were centrifuged and extracted to separate cell mass and analyzed further.

Biomass was determined by Dry Cell Weight (DCW) method.

Reducing sugar estimation was estimated by 3,5-Dinitrosalicylic acid (DNS) method as described by Miller [8].

Phenol sulfuric acid method given by Dubois was used for total sugar estimation [9].

Extraction

Broth was centrifuged: filtrate was taken and adjusted to pH 3.5 by concentrated HCl followed by addition of equal volume of ethyl acetate to the whole fermentation broth. Extraction was carried out on a rotary shaker at 220 rpm at ambient temperature for 6 h. The samples were subsequently centrifuged at 10,000 rpm for 15 min and the organic phase was collected for further steps [7,10].

Estimation of Lovastatin: HPLC Method

Estimation of lovastatin was done by HPLC method, which

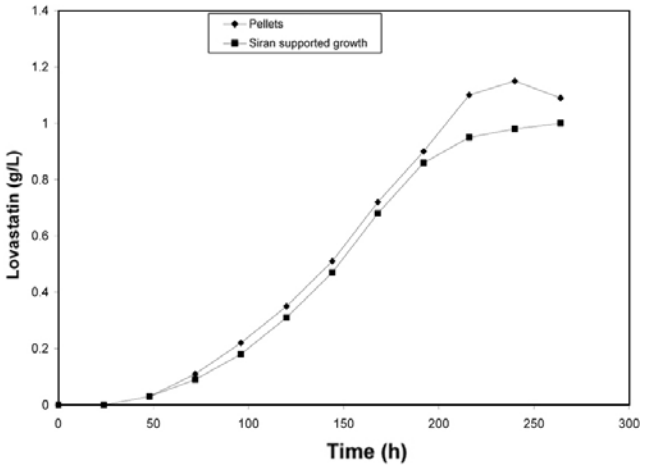

Fig. 1. Lovastatin concentration using pellets and siran supported growth of *A. terreus* with fermentation age.

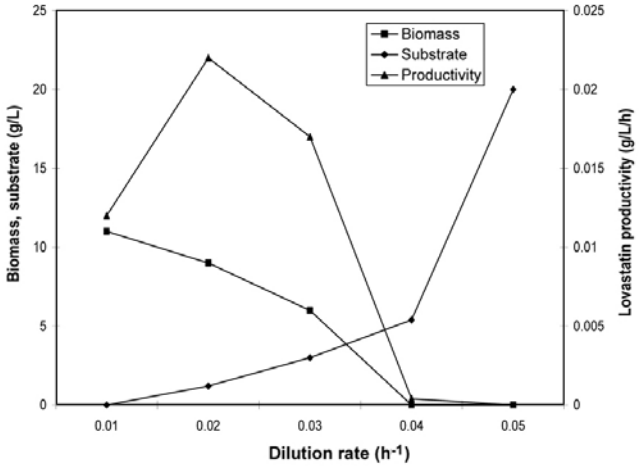

Fig. 2. Biomass, glucose, and lovastatin concentration during continuous mode of fermentation at different dilution rates using pellets.

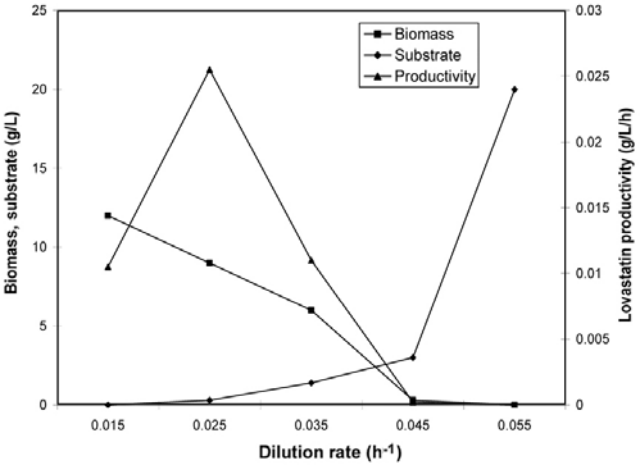

Fig. 3. Biomass, glucose, and lovastatin concentration during continuous mode of fermentation at different dilution rates using immobilized siran carriers.

was performed by using C18 column at 235 nm UV detector and 10 μL sample loop injector. Acetonitrile and water (60:40, v/v) was used as a mobile phase. The flow rate was maintained on 1.5 mL/min [11]. Reference solution was prepared using the standard lovastatin obtained from Lupin Limited, India.

RESULTS AND DISCUSSION

Study of Lovastatin Production Using *A. Terreus*

Lovastatin production using *A. terreus* pellets and siran carrier immobilized states was investigated in 5.0 L ALR. The batch fermentation in ALR was started initially using production medium under aseptic condition and samples were collected intermittently and analyzed. It was observed that after 9 days, pellets ruptured because of shear stress. A comparative profile of lovastatin production using pellets and immobilized siran carrier cells is depicted in Fig. 1. Lovastatin production starts after 48 h of fermentation and increase to a maximum level of 1.15 g/L by 240 h with pellets and with siran carrier it reached to 1.0 g/L only at the end of fermentation. The productivity of the immobilized siran supported growth of *A. terreus* fermentation was observed to be 0.0038 g/L/h, while that of pellets was 0.0048 g/L/h, which is higher than that of the siran supported growth. This may be due to the stress imposed by the immobilization process, unlike in pellet fermentation.

Effect of Airflow Rate on Lovastatin Production

Lovastatin fermentation is a highly aerobic fermentation. Aeration was supplied in ALR using sterile air. The effect of aeration rates (1.0~5.0 L/min) on the production of lovastatin with pellets and siran supported growth of *A. terreus* in an ALR was studied. Results are shown in Fig. 2 and Fig. 3. It is observed that at 1.0 L/min, the productivity was 0.008 g/L/h for pellet and 0.013 g/L/h for siran carrier. While an increase in aeration rate up to 3.0 L/min increased the lovastatin productivity up to 0.022 g/L/h for pellets and with siran supported growth productivity reached 0.0255 g/L/h. Further, increase in aeration rate resulted in decreased productivity due to attrition of siran supported growth and loss of viability of cells.

Effect of Dilution Rate on Lovastatin Production with *A. terreus*

Continuous mode of mold cultivation was carried out in 5.0 L internal loop ALR at a temperature of 28°C. The working volume in the continuous cultivation reactor was 4.0 L. Samples were analyzed for biomass, substrate, and lovastatin concentration. Initially the process in ALR was operated for 72 h in batch mode and then shifted to continuous mode keeping asep-

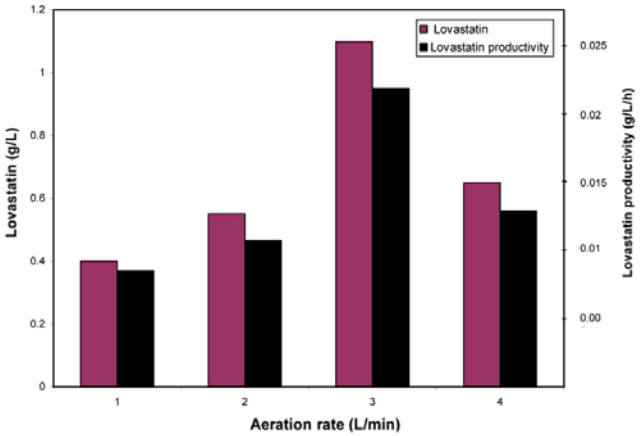

Fig. 4. Effect of airflow rate on lovastatin production using pellets.

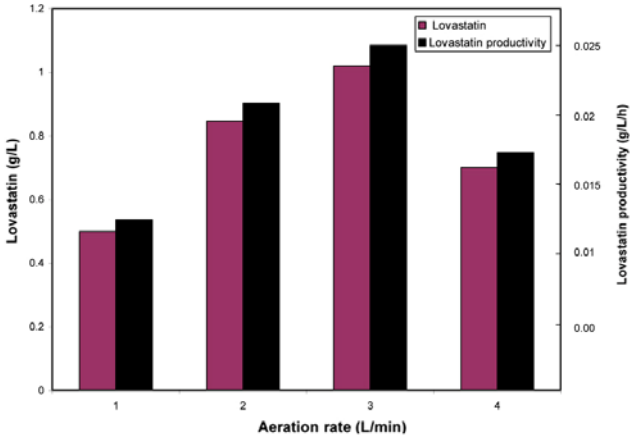

Fig. 5. Effect of airflow rate on lovastatin production using immobilized siran carriers.

tic conditions. The broth samples were collected every 24 h.

Figure 4 shows the profile of continuous lovastatin production in airlift reactor using pellets. Feeding of nutrients was carried out at a predefined dilution rates. The steady states were evaluated at various dilution rates from 0.01 to 0.055 h^{-1}. The process was started initially as a batch fermentation after which the batch cultivation was switched to continuous run, nutrient feed was commenced after 72 h for each dilution rate, and the optimal dilution rate for the continuous lovastatin production process was observed to be 0.02 h^{-1} with pellets.

In order to verify the possibility of improving the efficiency of continuous bioconversion, the effect of increasing the dilution rates were investigated during steady state growth in continuous culture. The objective was to determine an optimal dilution rate for lovastatin production. The continuous culture was operated at 5 different dilution rates between 0.01 to 0.05 h^{-1} when steady state lovastatin production was observed corresponding to a range of residence time from 20 to 100 h. However, with variation of the dilution rate the rate of uptake of nutrients and production of metabolites also vary.

An effective metabolite production is possible by optimizing the production rate in given conditions. The impact of dilution rate and residence time on lovastatin production with pellets of *A. terreus* was investigated. Experiments were conducted to evaluate the effect of dilution rate on growth by pellets of *A. terreus* in an ALR in continuous mode and the results are shown in Fig. 4. Results indicate that a decrease in lovastatin titer was observed with increasing dilution rates, but increasing the dilution rate from 0.01 to 0.02 h^{-1} productivity increases. Further increase in dilution rate resulted in decreased productivity. Dilution rate higher than 0.02 h^{-1} reduced the lovastatin content of culture broth, probably because the production of lovastatin, a secondary metabolite, was slow process; these higher dilution rates were too high in comparison to the rate of cell mass synthesized and biosynthesis of lovastatin. These results indicate that lovastatin production is non-growth associated. The data suggests that optimal lovastatin production can be obtained at a dilution rate of 0.02 h^{-1} in ALR for pellets and after 3 days of steady state lovastatin concentration settled to an average value of 1.1 g/L. The optimal lovastatin productivity was observed to be 0.022 g/L/h for pellets. The result suggests that to obtain high lovastatin production rate and yields, the dilution rate should be low. The cell washout limiting condition prevailed at a dilution rate of 0.04 h^{-1} for pellets.

With Siran supported growth, the lovastatin concentration reached to 1.02 g/L at dilution rate 0.025 h^{-1} and lovastatin productivity was observed to be 0.0255 g/L/h (Fig. 5). Hence immobilized siran supported growth of *A. terreus* can be used successfully for the continuous production of lovastatin, a secondary metabolite. It has also been observed that, at dilution rate, near to wash out condition, a sharp change in substrate concentration in the broth occurs. The cell washout limiting condition prevailed at a dilution rate of 0.045 h^{-1} for siran supported growth. The continuous fermentation profile suggests that there is subsequent utilization of the unutilized substrate or partially formed products, which could be used for further lovastatin production.

Continuous Production of Lovastatin in an ALR Using *A. terreus* Pellets

Continuous production of lovastatin with pellets was studied using optimized production medium in an ALR. Initially the airlift reactor was operated for 72 h in batch mode and then shifted to continuous mode. The reactor was operated for 35 days using pellets (Fig. 6). Broth samples were collected every 24 h and the effluent was discarded. Lovastatin production gradually decreased initially for 3 days; thereafter the amount of lovastatin production was maintained constantly for 18 days. But the concentration decreased later due to the development of sluggish mass cells. On Day 19th, feeding of production medium was stopped and the pellets were washed continuously with sterile saline solution at a flow rate of 120 mL/h for 24 h. Later, the bioreactor was fed with fresh production medium and the pellets were allowed to activate for 2 days. The rejuvenated cells were again used for continuous production of lovastatin. The process of activation was repeated on day 29th of continuous cultivation for pellets. As a

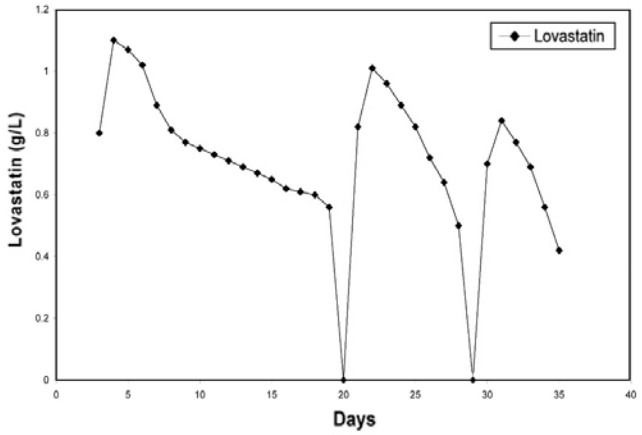

Fig. 6. Continuous production of lovastatin in an ALR using *A. terreus* pellets.

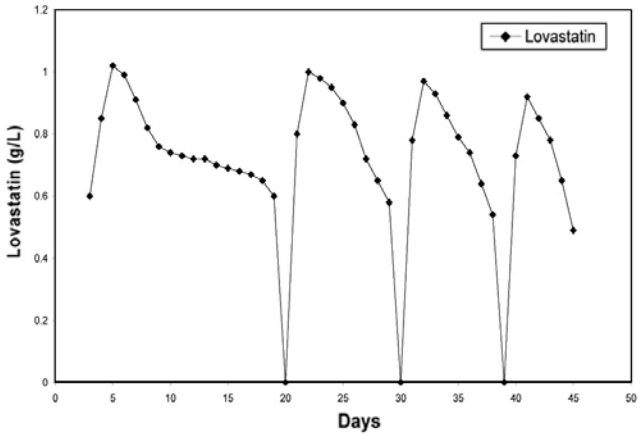

Fig. 7. Continuous production of lovastatin in an ALR using siran supported growth of *A. terreus*.

result, continuous cultivation for lovastatin production was carried out for 35 days using pellets and by intermittent treatment with saline solution.

Continuous Production of Lovastatin in an ALR Using Siran Supported Growth of *A. Terreus*

Continuous production of lovastatin with siran supported growth of *A. terreus* was studied using optimized production medium in an ALR. Initially the process was operated for 72 h in batch mode and then shifted to continuous mode. The reactor was operated for 45 days using siran supported growth (Fig. 7) without any attrition problems. Broth samples were collected every 24 h and the effluent was discarded.

Again the same process was carried out for 30 days and the bioreactor was fed with fresh medium. Further the process of activation was repeated on day 38 of continuous cultivation to carry out the process for 45 days. It is observed from the results that the immobilized cells of *A. terreus* on siran carrier

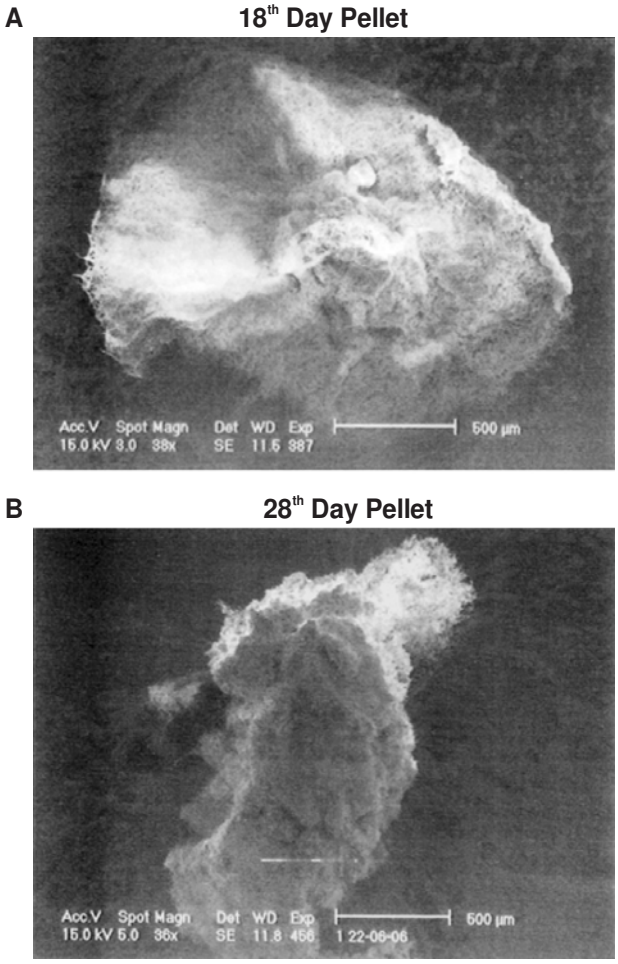

Fig. 8. Pellets during continuous production in air lift reactor.

are more efficient for the lovastatin production with continuous fermentation because cell viability remains for a longer period and the productivity is high. Also, lovastatin production using pellets gradually decreased with increase in operational time, this may be due to cell rupturing and decrease in cell viability due to severe shear stress caused by aeration.

A comparison of lovastatin production with pellets and immobilized cells by continuous fermentation processes is shown in the Table 1. The average productivity with continuous fermentation of siran supported growth of *A. terreus* (0.0255 g/L/h) was greater than with pellets (0.022 g/L/h). The total lovastatin produced with continuous fermentation was higher than with pellets. The overall results indicated that the continuous fermentation with immobilized siran carrier is more economical than the batch process in airlift conditions.

CONCLUSION

The results obtained in this work showed that lovastatin production is not associated with cell growth. It was observed that with siran carrier operational stability enhanced and the

Table 1. Comparison of lovastatin production with pellets and immobilized siran supported growth of *A. terreus* during continuous fermentation processes in an airlift bioreactor

Growth form of *A. terreus*	Total fermentation time (days)	Total production (g/L)	Productivity (g/L/h)
Pellets	35	1.1	0.022
Siran carriers	45	1.02	0.0255

process was carried out for 45 days with 0.0255 g/L/h productivity. After 45 days of continuous operation with immobilized siran carrier, the system did not show any decay which was observed for pellets after 28 days (Fig. 8) The application of continuous fermentation technique allowed good production for a working time as long as one and a half month without cell attrition. Dilution rates higher than 0.025 h^{-1} reduced lovastatin concentration in broth probably because lovastatin production is slow process. The data showed the increase in lovastatin productivity with siran supported growth of *A. terreus* and using pellets decrease in productivity was noticed. This is attributed to the mass transfer limitation of nutrients because of the barriers formed by immobilization technique. Also, the diffusion of product further slowed down because of these physical barriers. From the result, it may be concluded that immobilized cells of *A. terreus* on siran are efficient for production of lovastatin with continuous mode of fermentation.

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REFERENCES

1. Greenspan, M. D. and J. B. Yudkovitz (1985) Mevinolinic acid biosynthesis by *Aspergillus terreus* and its relationship to fatty acid biosynthesis. *J. Bacteriol.* 162: 704-707.
2. Manzoni, M., M. Rollini, S. Bergomi, and V. Cavazzoni (1998) Production and purification of statins from *Aspergillus terreus* strains. *Biotechnol. Techniques* 12: 529-532.
3. Srivastava, P. and S. Kundu (1998) A comparative evaluation of Cephalosporin C production using various immobilization modes. *J. Gen. Appl. Microbiol.* 44: 113-117.
4. Kumar, M. S., S. V. Kumar, and A. K. Sadhukhan (2000) Repeated fed batch process for improving Lovastatin production. *Proc. Biochem.* 36: 363-368.
5. Novak, K., S. Gerdin, and M. Berovic (1997) Increased lovastatin formation by *Aspergillus terreus* using repeated fed batch process. *Biotech. Letter.* 19: 947-948.
6. Seiskari, P., Y. Y. Linko, and P. Linko (1985) Continuous production of gluconic acid by immobilized *Gluconobacter oxydans* cell bioreactor. *Appl. Microbiol. Biotechnol.* 21: 356-360.
7. Samiee, M. S., N. Moazami, S. Haghighi, F. A. Mohseni, S. Mirdamadi, and M. R. Bakhtiari (2003) Screening of lovastatin production by filamentous fungi. *Biomed. J.* 7: 29-33.
8. Miller, G. L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* 31: 426-428.
9. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith (1956) Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28: 350-356.
10. Kysilka, R. and V. Kren, (1993) Determination of Lovastatin (mevinolin) and mevinolinic acid in fermentation liquids. *J. Chromatogr.* 630: 415-417.
11. Friedrich, J., M. Zuzek, M. Bencina, A. Cimermen, A. Strancar, and I. Radez (1995) High performance liquid chromatographic analysis of mevinolin as mevinolinic acid in fermentation broths. *J. Chromatogr.* 704: 363-367.