A Correlative Evaluation of Morphology and Rheology of *Aspergillus terreus* during Lovastatin Fermentation

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Abstract Lovastatin, a secondary metabolite, was produced by fermentation process using *Aspergillus terreus* in an internal loop airlift reactor. It is a highly aerobic fermentation process. Biomass concentration and cell morphology were evaluated and observed to contribute significantly to the high viscosity and pseudoplastic non-Newtonian behavior of the broth. Typical morphological changes over 10 days in the fermentation broth were studied. The viscosity increased from the start of the fermentation with an increasing cell mass content, reached to a maximum of 60 $N/m^2 \cdot s$ at 160 h and then declined after the branching of the hyphae with the formation of arthrospores. Rheological parameters like consistency index and fluidity index were evaluated. The consistency index was observed to increase from 9.8 to 66.85 N/m^2, while fluidity index decreased from 0.69 to 0.48 s^{-1} during 10 days of lovastatin production. A correlation between growth and consistency index of the broth has been evaluated. © KSBB

Keywords: lovastatin, Aspergillus terreus, *morphology*, *viscosity*, *consistency index*

INTRODUCTION

Lovastatin a secondary metabolite is a potent hypocholesterolemic agent, generally produced by fermentation process using *Aspergillus terreus* [1,2] during the secondary phase of the growth of microorganism, the idiophase that follows the trophophase. *A. terreus* is a filamentous fungus which, grown in agitated liquid culture, typically forms pellets. The pellets are formed by spore aggregation immediately before and during the germination.

Fungal morphology is an important parameter, which influences the physical properties like viscosity and density of the fermentation broth. Generally, two growth forms, the filamentous and the pelleted form can be observed in a submerged fungal fermentation, the filamentous form being more viscous than pelleted form [3]. Fungal broth exhibits highly viscous non-Newtonian behavior and is represented by the power law model. Biomass concentration and cell morphology contribute significantly to the high viscosity and pseudoplasticity of the fermentation broths. Variation of the broth characteristics affects the bioreactor hydrodynamic properties which include mixing and mass transfer performances [4-6]. This impact on the transport processes in a bioreactor has a strong influence on the efficiency and productivity of entire fermentation process.

Lovastatin production is an aerobic fermentation process with a wide variation of oxygen mass transfer in the broth. The morphological differentiation of the fungus is a prerequisite for the production of secondary metabolite.

The present study evaluates the broth viscosity in relation to morphological variation during the growth of the microorganism and correlates it to the rheological properties of the broth during the production of lovastatin in airlift bioreactor. The dissolved oxygen tension was throughout not less than 40% of air saturation.

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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, IL, USA. It was

maintained on PDA incubated at 28°C for 4~5 days and stored under refrigeration at 5~10°C.

Growth media [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, 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, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 5 mg/L. The pH of media was adjusted to 6~6.5 with HCl, before sterilization and the fermentation was carried out on a rotary shaker at 220 rpm, 28°C for 3~4 days.

The culture was grown in 500 mL shake flask containing 100 mL medium. Large numbers of floccs (pellets) attaining the size of less than 1 mm to several millimeters were formed. Morphology of different pellets was characterized by measuring the projected area, mean diameter and circularity of pellet. The various pellet sizes were observed in growth media: 1.6~1.8 mm 40%, 2.0~2.4 mm 50%, and 2.6~2.8 mm 10%. The average size of the pellet ranging from 1.8 to 2.0 mm was used to inoculate the production media for lovastatin.

Smaller pellets gave poor productivity of lovastatin as filament zone (lovastatin producing) and core region (oxygen containing) are very less in size. The larger pellets were internally hollow due to the necrosis of the fungal biomass caused due to the lack of oxygen inside the pellet, which also showed lower lovastatin production. A high concentration of oxygen is essential to attain a high titer of lovastatin [8].

Thus an intermediate size which is less dense, open, filamentous pellet, ranging from 1.8 to 2.0 mm diameter; with a high ratio of filament-to-core zone, grown in an oxygen rich environment were used for maximal production of lovastatin.

Production media contained 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, 28°C for 10 days.

Experimental Set-up

A 2.0-liter internal loop airlift reactor (ALR) made of borosilicate glass was used to study the production process. A constant airflow rate was maintained through an air filter and was measured with a rotameter. All the ports of ALR were aseptically sealed after inoculation. Sterile air was sparged co-currently through a single nozzle sparger. Batch fermentation was carried out at 28°C at pH 6.5 at an aeration rate of 3.0 L/min. Several samples were collected, centrifuged and analyzed. The schematic design is shown in Fig. 1.

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.

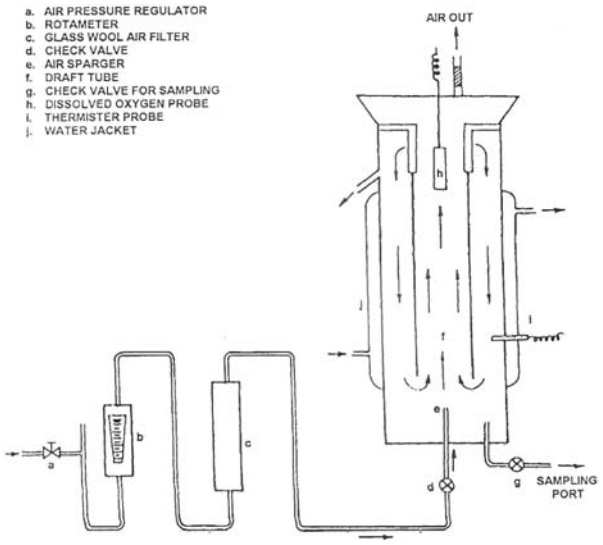

Fig. 1. The schematic diagram showing the ALR.

Biomass was determined by dry cell weight (DCW) method. Reducing sugar was estimated by 3, 5-dinitrosalicylic acid (DNS) method [9]. Phenol sulphuric acid method was used for total sugar estimation [10].

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 [7,11].

Estimation of Lovastatin

Estimation of lovastatin was done by HPLC method, which 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 [12].

Reference solution was prepared using the standard lovastatin obtained from Lupin Limited, India.

Image Analysis for Cell Morphology

Morphological changes that occur during growth and production of lovastatin were studied with B-1 series system microscope connected with Motic Images Plus 2.0 ML software and with Scanning Electron Microscopy. The morphology of pellets was characterized by their mean area and mean diameter. The specimens were observed and photographed with a Philips XL 20 scanning electron microscope.

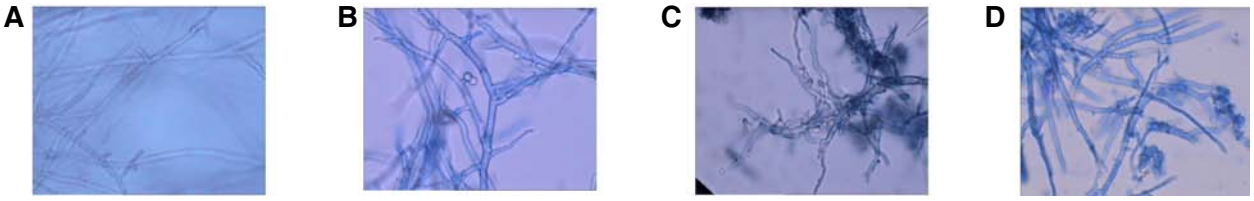

Fig. 2. Morphological changes during 10 days fermentation process for lovastatin. (A) 3rd day of lovastatin production, (B) 5th day of lovastatin production (arthrospores), (C) 7th day of lovastatin production, (D) 9th day of lovastatin production.

The accelerated voltage was 15 kV, and beam specimen angle was 45°.

Fluid Dynamics Measurements

Broth Rheology

Off-line viscosity measurements were used to obtain values of consistency index factor (k) and the flow behavior index (n). A Brookfield viscometer (Dial Reading and Digital DV-I, No. M/85-170-B) was employed for viscosity measurements.

The consistency factor and flow behavior index were calculated from the logarithmic shear stress (τ) versus shear rate (γ) plot. An apparent viscosity (μ_{ap}) was estimated using Ostwald-de Waelle power law approach for non-Newtonian fluids as shown below:

$$\mu_{ap} = \tau / \gamma = k\gamma^{n-1}$$

Evaluation of Biomass Exponent α

The influence of biomass concentration on broth rheology was evaluated by taking large volume of samples throughout the several fermentations of large batch and this was reconstituted to large biomass sample using fermentation media 5~6 sub samples of identical morphology with biomass concentration ranging between 10~40 g/L as in Tucker and Thomas [13].

From the rheological measurements on these sub sample values of the biomass exponent α (applied to consistency index) at various time could be estimated. The α values were determined from the slope of logarithmically transformed plots of consistency index vs sub sample biomass concentration (α being the slope of plotted line). A globally applicable α value for consistency index correlation was determined by taking mean α value throughout the time of fermentation. Standard deviation and standard error for α value were also estimated.

RESULTS AND DISCUSSIONS

Growth and Morphological Changes

Batch fermentation run were performed in 2.0-liter ALR for 10 days. Samples were collected intermittently and studied for cell morphological characteristic variation during the growth and production phase of *A. terreus* (Fig. 2). The early growth phase (trophophase) was followed by late lovastatin production phase (idiophase), which starts after 150 h and extends till 240 h of fermentation. In the later fermentation stages the growth ceases.

The morphological variation during the growth of *A. terreus* has been evaluated by microscopy of the cells of the broth. It was observed (Fig. 2) that typical morphological changes occurred over 10 days in the fermentation broth. In the early fermentation stage the slender filamentous hyphae exist. As the growth proceeds to 3rd day hyphae grows thicker, stout and shows branching pattern. As well as 4th day onwards, the thick hyphae developed into swollen hyphal fragments, the number of tips rapidly increases, the hyphal length is shortened and the cell thickness increases. Some spherical and swollen arthrospores were also observed in fermentation broth and their number started increasing from the 5th day. From 6th day until 9th day the large numbers of arthrospores were observed. However, the remaining hyphae became slender and broke into smaller fragments.

It is observed that with the formation of arthrospores there is an increase in lovastatin production, which extends till the 10th day. The lovastatin production increased significantly in this period. It is postulated that with the increase in the age of fermentation enhancement of the branching frequency, the productivity of mycelium increases.

It was observed that the mean length of freely dispersed mycelia tend to increase in early stages of growth from 24~70 h, which depicts that the breakage rate was lower than the growth rate. During the deceleration phase, (90~140 h), the breakage rate was greater than the growth rate, which led to a decrease in the length of mycelia. The rate of breakage was highest during this period of fermentation.

Earlier studies have shown [14], breakage should predominate during biomass decline phase (after 160 h) and our experimental data substantiate it. It is depicted that during the decline phase *i.e.* from 160 h onward the breakage was less in comparison to that occurs in deceleration phase. It is suggested that a decrease in the size of mycelia during the deceleration phase also leads to a decrease in the disruptive forces.

It can be depicted that during the deceleration phase the larger mycelia started to break up gradually. Growth was negligible compared with breakage in this period and the net effect was narrowing of the mycelia size distribution (*e.g.* major axis), which caused a decrease in the standard deviation of size distribution (data not shown). It is postulated that the mycelia are likely to have a distribution of mechanical

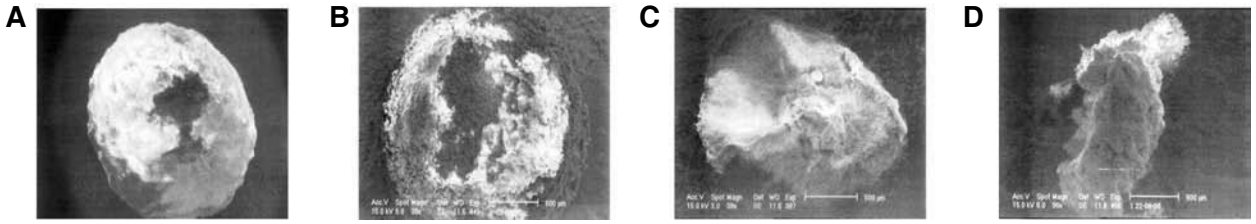

Fig. 3. SEM result showing change in the pellet structure during 10 days of production of lovastatin fermentation. (A) 4th day pellet, (B) 6th day pellet, (C) 8th day pellet, (D) 10th day pellet.

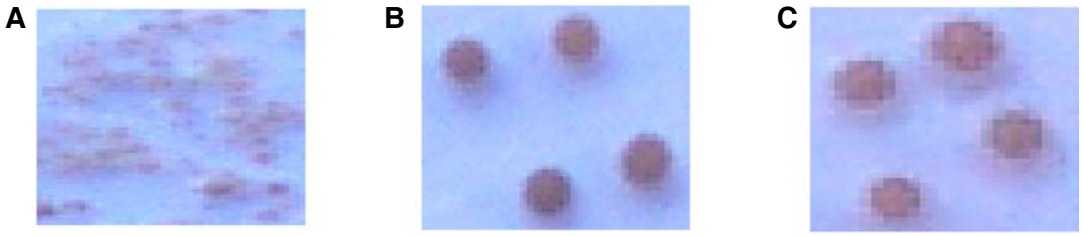

Fig. 4. Pellets of various size groups. (A) Pellet size ranging from 1.8~2.0 mm, (B) Pellet size ranging from 3.4~3.6 mm, (C) Pellet size ranging from 5.0~5.4 mm.

strength and some of them that survive the imposed shear forces are physically strong and continue to grow at the end of the breakage period (*e.g.* small mycelia were lysed and a percentage of the rest continued to grow resulting in widening of the size distribution). Therefore, it may be postulated that cell lysis during this period was accompanied by some cell re-growth.

Scanning Electron Microscope Studies

In batch fermentation, the broth was subjected to lower agitation speed in shake flask, to obtain pellet/floccs. These pellets were transferred to 2.0-liter ALR for lovastatin production studies.

Pellet morphology was characterized with respect to diameter, circularity, roughness, and compactness (Fig. 3). After the formation of mycelial clumps, pellets with a spherical and dense core appeared in the culture. However, as the fermentation proceeded, the outer hairy region of the pellets became fluffier and there was a corresponding increase in pellet circularity and roughness. The cells formed mainly pellets and their size increased continuously during the entire culture period. The pellets of various size groups are shown in Fig. 4.

The pellet diameter increased rapidly from the first day to the end of fermentation (Table 1). The roughness of the pellets continuously increased throughout the entire fermentation period.

At the later stages of fermentation (6th day), the circularity and compactness of the pellets at 3 L/min of aeration was decreased. The circularity means a shape factor describing the deviation of the pellet image from a true circle and roughness means the irregularity of the perimeter of the pellet, compact pellet formation with delayed pellet deformation.

Table 1. Showing variation in pellet size and specific growth rate during 10 days fermentation of lovastatin

	No. of days	Pellet size (mm)	Specific growth rate (h^{-1})
1	3rd day	3.4~3.6	13.2
2	5th day	5.0~5.4	10.0
3	7th day	6.3~6.6	8.3
4	9th day	5.5~5.8	6.9

As the fermentation proceeded (8th day), the outer hairy region of the mycelial pellets formed at high aeration intensity (3 L/min) was markedly shaved off and the core area was reduced, resulting in a corresponding decrease in pellet diameter and compactness.

After 10 days, pellet break-up was most likely caused by cell lysis within the pellets, resulting in the loss of stability of the pellet. At later stages of fermentation, typically after 8th day, the pellets not only broke up but the mycelia lost its rigidity and appeared somewhat withered.

Pellet roughness decreased rapidly following step-wise increase in the fermentation age, which may be due to attrition of the pellets. However, this does not always indicate formation of more compact pellets by the fragmentation of the outer, relatively less entangled regions because the compactness of the pellet at high agitation intensity indicated a low value due to the formation of a low core area.

Lovastatin Production

For most fungal fermentations, productivity of metabolite is dependant on morphology. The size of pellet is very important for the formation of secondary metabolites. A mean

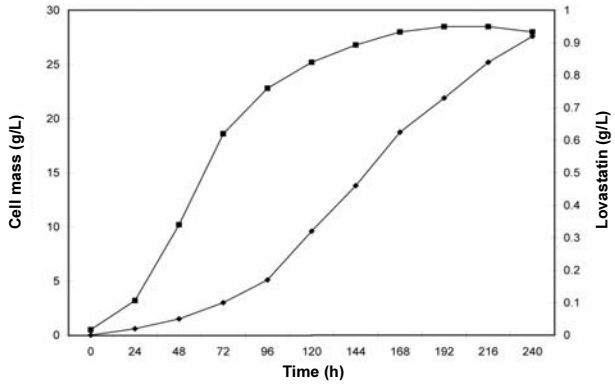

Fig. 5. Graph showing change in biomass concentration and lovastatin concentration with fermentation time. —■—; Cell mass, —◆—; lovastatin.

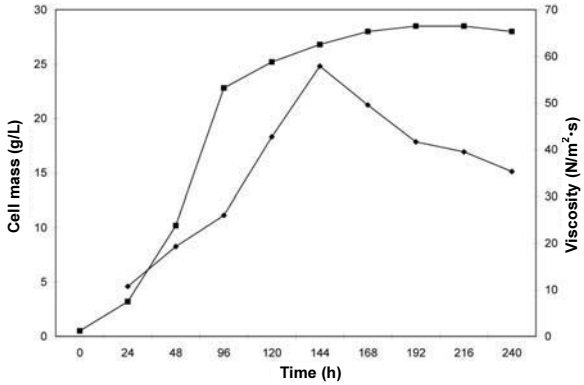

Fig. 6. Graph showing variation of cell mass and viscosity with fermentation time. —■—; Cell mass, —◆—; viscosity.

pellet size of 1.8 to 2.0 mm is preferred for lovastatin production.

During batch fermentation process in ALR, the aeration rate was 3.0 L/min. The lovastatin production started at 48 h onwards and reached to a maximum of 0.92 g/L at 240 h (Fig. 5). The product formation was quite rapid in the stationary phase of fungal morphology. The kinetic analysis showed a maximum specific growth rate of 0.025 h^{-1} in the early trophophase followed by decreased growth rate 0.0017 h^{-1} at the end of fermentation.

Lovastatin production rate increased sharply after 72 h and extended till 170 h, which coincides with the declining growth rate. The specific product formation rate was 1.35×10^{-4} h^{-1}.

Rheological Properties of Fermentation Broth

Samples were collected in triplicate during batch fermentation of lovastatin production. The broth was evaluated for cell mass and offline viscosity measurement. It was observed that broth exhibits a typical pseudo plastic non-Newtonian behavior and follow power law model. The viscosity of the broth increased in the early hours of fermentation and reached to maximum at about 150 h, which might be attributed to the growth phase of the microbes. A maximum biomass concentration of about 28 g/L was observed at 168 h. As seen in microscopy the mycelium changed into thick/stout in structure. The change in the apparent viscosity of the broth with cell mass concentration depicts the enhancement of viscosity with morphological differentiation of *A. terreus.* In the later fermentation stages, after 150 h, viscosity of the broth was reduced (Fig. 6). It may be attributed to the variation of morphological features of the mycelia, which changes into arthrospores that are spherical in shape and thus behave as a suspension of spherical structures.

A sharp fall in the D.O. level was observed during the rapid exponential growth phase up to 144 h. However with the formation of arthrospores after the 5th to 6th day, the fall in D.O. level decreased and D.O. was maintained at 40% satu- ration (data not shown).

Viscosity variation can be correlated to the change in cell mass of the broth. The rheological property can be correlated according to, Tucker correlation [15]. This defines the correlation of rheological parameters to the biomass concentration with an experimental correlation constant α (biomass exponent) as an exponent. Fig. 6 shows that the viscosity increased (from the start of the fermentation) with the increasing cell mass content, reaches to a maximum of about 60 $N/m^2 \cdot s$ at 150 h and then declined. The decrease in viscosity may be attributed to the differentiation of swollen hyphae fragments into arthrospores. The increase in hyphae thickness led to clump formation, which may be further correlated to compactness and roughness. The consistency index (k) and fluidity index (n) were also evaluated. It was observed that k initially increases rapidly with the biomass from 9.78 N/m^2 at 48 h to 66.85 N/m^2 at 168 h at 28 g/L cell mass content and the values of n were observed to decrease from 0.694 to 0.48 s^{-1} (Fig. 7).

α value was determined for the correlation of consistency index to biomass concentration is shown in Fig. 8. The mean α value was approximately 2.0, the standard deviation was 0.088 and the standard error of mean was 0.039. This is quite similar to the other studies by Tucker. This suggests that the difference observed between the measured and the predicted parameter values might be derived by degradation of biomass. It can be further proposed that mycelial degradation might affect hyphal rigidity and clump interaction causing the change in the exponent on the biomass concentration, α.

The consistency index divided by biomass concentration to the power of α (k/cm^{α}), plotted against fermentation time for the five fermentations shown in Fig. 9. This illustrates the reason for the poor quality of correlations based purely on biomass concentration. Fig. 9 clearly shows that k/cm^{α} is equivalent to this constant, and changes throughout the fermentations. A relationship between the 'changing constant', or rather k/cm^{α} and a morphological factor should be sought.

This broth exhibits a typical non-Newtonian system, wherein it is composed of both liquid and solid particles (spherical hyphal mass and arthrospores). The profile of k/cm^2 vs time (Fig. 9) and profile of n/cm^2 vs time (Fig. 10) shows a decline in the early fermentation hours, followed by

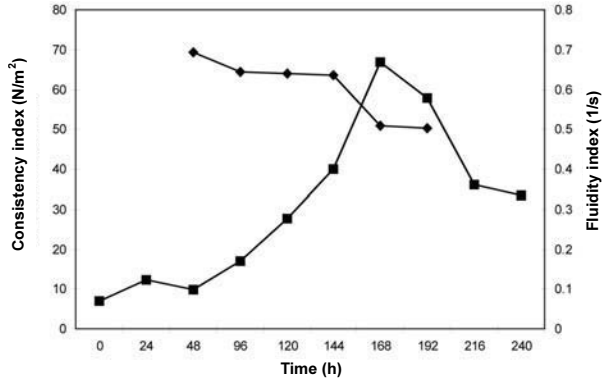

Fig. 7. Change in broth properties with time. —■—; consistency index, —◆—; fluidity index.

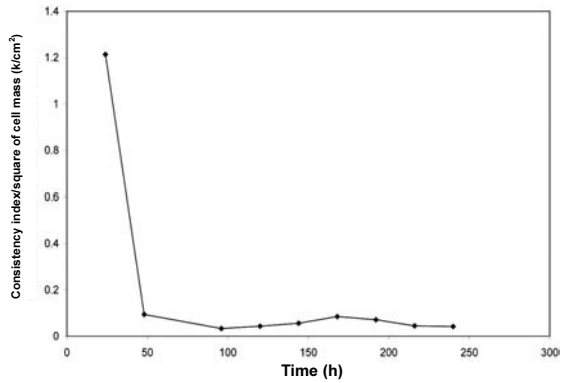

Fig. 9. Time profile of the consistency index, *k* divided by squared biomass concentration as dry cell weight for fermentation. —◆—; k/cm^2.

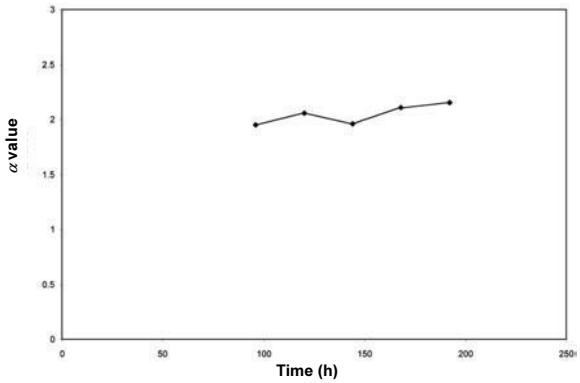

Fig. 8. Logarithmic plot of consistency index vs time for evaluation of exponent of biomass (α value determination).

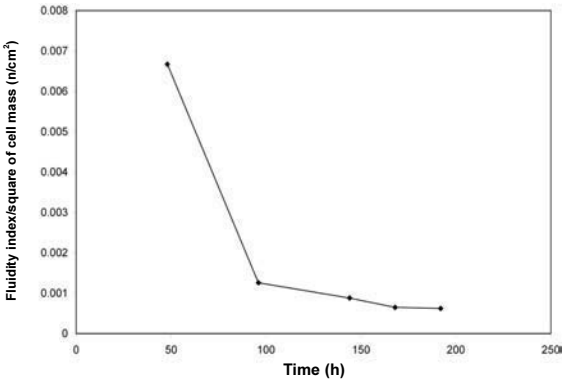

Fig. 10. Time profile of the fluidity index, *n* divided by squared biomass concentration for fermentation. —◆—; n/cm^2.

a low constant value that is quite similar to an observation for cephalosporin-C fermentation [16]. Also the result depicts the reason for poor correlation with the biomass concentration, as k/cm^2 and n/cm^2 deviates throughout the fermentation. Hence it is imperative to develop a relationship between the morphological factor and k/cm^{α}.

Broth Density Study

An increase in biomass concentration causes an increase in phase volume (volume of suspended material/volume of continuous phase) that ultimately causes an increase in apparent viscosity.

Broth density varies with age of fermentation. In the early fermentation hours, the broth showed watery fluid nature. The density increases as the fermentation proceeds and reaches up to 1.2 g/mL at 168 h. With subsequent increase in time, no further increase in broth density was observed. However, the broth density decreased slightly at the end of the fermentation.

The colloidal forces between the mycelia as well as the mycelial entanglement define the interaction between the mycelia and hence affect the phase volume. Assuming mycelial interactions are important for lovastatin production, therefore the particle size as well as its distribution is for the rheology of the broth.

CONCLUSION

The present study on morphological variation during lovastatin production in airlift reactor suggests a direct correlation of cell morphology, rheological characteristics and lovastatin production. The rheological studies using *A. terreus* broth exhibit a typical fungal fermentation and follow power law model. Free cell fermentation exhibit enhanced viscosity with the age of fermentation, which may control the oxygen transfer phenomenon and finally lovastatin productivity in the broth. Lovastatin production rate decreases with the age of fermentation. It is observed that consistency index is strongly correlated with the biomass concentration (biomass exponent). The α value correlates the morphology to viscosity and the value is observed to be 2.0 and is constant throughout the fermentation. Use of average α value enables the influence of morphology to be examined independently of biomass concentration. The value of k/cm^{α} is observed to be changing throughout the fermentation. This

confirms that the use of correlation to predict the rheological characteristics of broth that based only on biomass concentration is not correct. However no correlation has been developed with fluidity index with morphology or biomass concentration.

The morphology of *A. terreus* growth has been characterized. The pellet of intermediate, hairiness and roughness was necessary for better production of lovastatin. It was found that roughness and circularity decreases rapidly in the later stages of fermentation, which may be due to attrition of the pellets. The study of oxygen mass transfer variation with time needs to be further evaluated.

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NOMENCLATURE

ALR	Airlift reactor
D.O.	Dissolved oxygen
SEM	Scanning electron microscope
k	Consistency index factor
n	Flow behavior index
γ	Shear rate
μ_{ap}	Apparent viscosity

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