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Enhanced production of lovastatin in a bubble column by *Aspergillus terreus* using a two-stage feeding strategy

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Abstract: A two-stage feeding strategy is shown to improve the rate of production of lovastatin by *Aspergillus terreus* when compared with conventional batch fermentation. The feeding strategy consisted of an initial batch/fed-batch phase and a semi-continuous culture dilution phase with retention of pelleted biomass in a slurry bubble column reactor. The batch phase served only to build up the biomass for producing lovastatin, a secondary metabolite that inhibits its own synthesis in the producing microfungus. The semi-continuous dilution phase provided nutrients to sustain the fungus, but prevented biomass growth by limiting the supply of essential nitrogen. (Synthesis of lovastatin does not require nitrogen.) The preferred pelleted growth morphology that favors lovastatin synthesis was readily obtained and maintained in the 20 L bubble column used. In contrast, a stirred tank fermentation had a substantially lower production of lovastatin because mechanical agitation damaged the fungal pellets. The two-stage feeding method increased lovastatin production rate by more than 50% in comparison with the conventional batch operation. Rheological data for the fungal broth are presented.

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Keywords: lovastatin; *Aspergillus terreus*; bubble column; rheology; semi-continuous

NOTATION

B-I, B-II	Batch fermentation runs
C_{lov}	Concentration of lovastatin at time t (kg m^{-3})
D	Dilution rate (s^{-1})
g	Gravitational acceleration (m s^{-2})
K	Consistency index of broth (N m^{-2} s^{n})
l	Length scale of micro-eddies (µm)
n	Flow behavior index of broth
r_{lov}	Rate of production of lovastatin (kg s^{-1})
SC-4–7	Semi-continuous fermentation runs
STR	Stirred-tank fermentation run
t	Time (s)
U_G	Superficial gas velocity in the bubble column (m s^{-1})
μ_L	Viscosity of broth (Pa s)
ρ_L	Density of broth (kg m^{-3})

INTRODUCTION

The cholesterol-lowering drug lovastatin is commercially produced by submerged culture of the filamentous fungus *Aspergillus terreus*.[1,2] Fermentations are usually carried out in stirred tanks in batch mode. Maintaining a high concentration of dissolved oxygen is essential for attaining a high titer of lovastatin.[3]

As a secondary metabolite, lovastatin generation starts at the onset of the stationary phase and lasts around 10 days. Lovastatin synthesis is self-inhibitory[4] and this effect limits culture productivity in batch mode of fermentation. As an alternative to batch culture, a fed-batch strategy has been attempted.[5,6] Novak *et al*.[6] observed that in fed-batch fermentations synthesis of lovastatin could be prolonged compared with batch fermentation, but productivity was reduced. Kumar *et al*.[5] reported more encouraging results in stirred-tank fed-batch culture of an overproducing strain of *A. terreus*.

Here we report a culture strategy involving an initial batch/fed-batch phase to build up the biomass, followed by a semi-continuous culture phase at a dilution rate of 0.26 d^{-1} in a slurry bubble column that retains the biomass pellets within the reactor. Use of a bubble column bioreactor prevented mechanical damage to fungal pellets, which has been observed to reduce lovastatin productivity in conventional stirred-tank fermentations of *A. terreus*.[7,8] The relationships among culture morphology, broth rheology and lovastatin production are reported for semi-continuous operation. Results are compared with the literature data reported for stirred-tank culture of the same fungus with the same medium as used in the present work. To our knowledge, usefulness of this type of culture strategy has not been assessed for other fermentations. Other factors that influence lovastatin production include the carbon:nitrogen ratio in the medium and the level of dissolved oxygen.[3,4]

MATERIALS AND METHODS

Microorganism and inoculation

Aspergillus terreus ATCC 20 542 was obtained from the American Type Culture Collection. The fungus was maintained in Petri dishes of potato dextrose agar.

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After inoculation from the original slant, the dishes were incubated at 28 °C for 5 days and subsequently stored at 5 °C. A suspension of spores was obtained by washing the Petri dish cultures with a sterile aqueous solution of 2% Tween 20. The resulting suspension was centrifuged (∼2800 × *g*, 5 min) and the solids were resuspended in sterile distilled water. The spore concentration was determined spectrophotometrically at 360 nm. A standard curve was used to correlate the optical density to direct spore counts, which had been made with a flow cytometer (Coulter Epics XL-MCL, Beckman Coulter Ltd, High Wycombe, UK).

Growth conditions

Fungal pellets were obtained by germination from spores suspended in shake flasks in a preliminary fermentation stage and used for further inoculation of the bubble column bioreactor operated at 28 °C. Seed cultures were carried out in 1000 mL flasks containing 250 mL of medium, held on a rotary shaker at 150 rpm, 28 °C for 48 h. Fermentations lasted around 10 days. The culture medium contained lactose as a carbon source and soybean meal as a nitrogen source. The medium contained (per liter): 114.26 g lactose, 5.41g soybean meal, 0.8 g KH_2PO_4, 0.4 g NaCl, 0.52 g $MgSO_4.7H_2O$, 1 mg $ZnSO_4.H_2O$, 2 mg $Fe(NO_3)_3.9H_2O$, 0.04 mg biotin, and 1 mL of a trace element solution. The trace element solution contained (for 1 L of solution): $Na2B4O_7.10H_2O$, 100 mg; $MnCl_24H_2O$, 50 mg; $Na2MoO_4.2H_2O$, 50 mg; and $CuSO_4.5H_2O$, 250 mg. The initial pH was adjusted to 6 with 0.1 mol L^{-1} NaOH.

Bubble column bioreactor

Fermentations were conducted at 28 °C in a 20 L (17 L working volume) slurry bubble column bioreactor with an aspect ratio of 6 (Fig. 1). The diameter of the reactor vessel was 0.155 m. Gas was sparged through a perforated plate (150 holes of 1.5 mm diameter) located at the base of the reactor. Gas flow rate was held constant at 1.0 vvm in the various runs. The reactor was fitted with a jacket for temperature control. The top degassing zone of the column had a jacket of its own and this was cooled at 4 °C to prevent wall growth (Fig. 1). Experiments were conducted with oxygen-enriched air (80% oxygen) as the sparged gas. Dissolved oxygen concentration in the liquid was controlled at 400% of air saturation. The gas leaving the reactor was recirculated in a closed loop. Prior to recirculation, carbon dioxide was removed by absorption in a $Ba(OH)_2$ solution and pure oxygen was added to maintain the specified oxygen level. Absorption in barium hydroxide ensured that the carbon dioxide concentration in the sparged gas remained at <0.1% v/v CO_2. Oxygen and CO_2 partial pressures in the gas stream were measured with online sensors (Adaptive Biosystems Ltd, Luton, UK).

The culture methodology consisted of three different phases, as follows: (1) a batch phase that lasted 4, 5 or 7 days for the runs designated as SC-4, SC-5 and SC-7, respectively; (2) a fed-batch phase that commenced after the batch phase. During this phase, approximately 2.4 L of medium was added over the course of 12 h until the level reached the harvest port; (3) a semi-continuous culture phase that commenced as soon as the broth level reached the harvest port. Although there were three different phases, this operational mode is considered a two-stage feeding strategy because the fed-batch phase was quite short compared with the other two phases (i.e., batch and semi-continuous). During the fed-batch and the semi-continuous culture phases, fresh medium was fed using a diaphragm pump through a port located on top of the reactor. The feed medium had the same composition as specified in the previous section except that the nitrogen source (i.e., soybean meal) was not present. The dilution rate was 0.26 d^{-1}. The feed pump was calibrated before each experiment. The withdrawal of the medium was by gravity overflow. The entrance of the overflow pipe had a wire mesh (1.0 mm diameter holes) installed. During the batch and the fed-batch

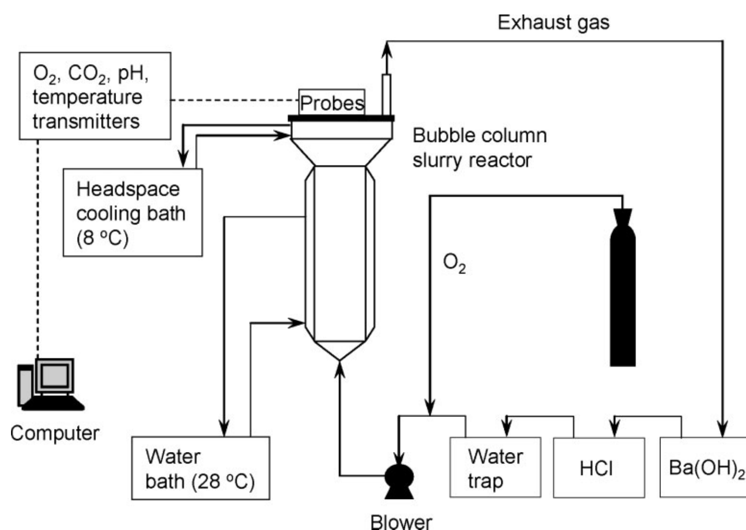

Figure 1. Bioreactor set-up.

phases that preceded all semi-continuous cultures, the working level was maintained lower than the location of the mesh screen (13.8 L working volume during batch phase), to prevent its clogging by fungal growth.

Rheological measurements

Rheological parameters (K, n) were measured using a programmable rotational viscometer (Brookfield DV-II+ with standard vane spindle V-72, 21.7 mm diameter, 43.3 mm height; Brookfield, Middleboro, MA, USA). All measurements were carried out at 28 °C in a glass vessel of 35 mm diameter, filled to 70 mm, following the method described in Casas López *et al*.[7]

Morphological measurements

Fungal pellet morphology was characterized using image analysis.[9] Prior to imaging, each sample of the fermentation broth was processed as follows. 10 mL of sample was decanted and washed twice with 20 mL of distilled water. Within a sample, 100 objects were analyzed for each determination. The image was captured with a CMOS camera (Evolution LC Color, Media Cybernetics, Inc., Silver Spring, MD, USA) mounted on an inverted microscope (Leica DMIL, Leica Microsystems GmbH, Wetzlar, Germany) that used a ×40 magnification. Image analysis was performed with the software package Image-Pro Plus 4.5.1 (Media Cybernetics, Inc.).

Changes in pellet morphology were quantified using the following two measures: (1) the diameter corresponding to a circular area equivalent to the total pellet projected area, as a one-dimensional measurement of the pellet size; and (2) the ratio between the area of the peripheral 'hairy surface' and the total area of the pellet. This ratio was termed the 'filament ratio'. These two measures provided a direct indication of the pellet size and the proportion of filamentous growth in it. For instance, in the early stages of fermentation, a young pellet would be typically characterized by a small diameter and a filament ratio close to 100%. As fermentation progressed, the filament ratio would reduce.

Analytical methods

Biomass

The biomass (as dry weight) was determined by filtering a known volume of the broth through a 0.45 µm Millipore cellulose filter, washing the cells with sterile distilled water, and freeze-drying the solids.

Lovastatin

Lovastatin was measured in its β-hydroxyacid form by high-performance liquid chromatography (HPLC) of the biomass-free filtered broth.[10,11] The filtered broth containing the β-hydroxyacid was diluted 10-fold with acetonitrile–water (1:1, v/v) prior to analysis. Pharmaceutical-grade lovastatin (lactone form) tablets (Nergadan tablets, J Uriach and Cía, Barcelona, Spain) were used to prepare the standards for HPLC analyses. Prior to use, the lactone form was converted into its β-hydroxyacid form by dissolving the tablets in a mixture of 0.1 mol L^{-1} NaOH and ethanol (1:1, v/v), heating the solution at 50 °C for 20 min, and neutralizing it with HCl. HPLC was done on a Beckman Ultrasphere ODS (250 × 4.6 mm I.D., 5 µm) column. The column was mounted in a Shimadzu model LC10 liquid chromatograph equipped with a Shimadzu MX-10Av diode array detector (Shimadzu Corp., Kyoto, Japan). The eluent was a mixture of acetonitrile and 0.1% phosphoric acid (60:40, v/v). The eluent flow rate was 1.5 mL min^{-1}. The detection wavelength was 238 nm. The sample injection volume was 20 µL.

Nitrogen

Total nitrogen concentration in the culture samples was measured by a total organic carbon analyzer TOC-VCPN (Shimadzu Corp.) equipped with a total nitrogen unit (Shimadzu Corp.). The nitrogen in the samples was analyzed by catalyzed oxidation by combustion at 720 °C and luminescence analysis of the gas produced. The samples had been previously filtered with a 0.45 µm Millipore cellulose filter and diluted with Milli-Q deionized water.

RESULTS AND DISCUSSION

Biomass concentration profiles for the semi-continuous runs SC-4, SC-5, and SC-7 are shown in Fig. 2. Irrespective of the duration of the batch phase – i.e., 4, 5, and 7 days for runs SC-4, SC-5, and SC-7, respectively – the biomass growth profiles were quite similar (Fig. 2). Furthermore, the growth profiles for runs SC-4–7 were comparable to those for runs B-I and B-II, which exclusively involved batch operation for the entire duration of the fermentation[12] (runs B-I and B-II were duplicate batch runs that were identical in every respect). Thus, as intended, the fed-batch and semi-continuous flow operations did not cause biomass growth beyond the level that had been attained in the batch phase. This was because the feed medium did not have nitrogen. The feeding served only to sustain the fungus and dilute the lovastatin produced so that self-inhibition[4] of its synthesis could be reduced. The average final biomass level attained in the three semi-continuous runs was $9.1 \pm 0.3\,g\,L^{-1}$.

The measured nitrogen concentration data points during semi-continuous fermentations are shown in Fig. 3. In all cases, the nitrogen level by day 4, i.e., the start of the earliest feeding, had declined to 26% or less of the initial nitrogen concentration of 460 mg L^{-1}. The solid lines N4–7 in Fig. 3 are the calculated profiles of nitrogen concentration. These calculated profiles are based on a nitrogen mass balance at various stages of fermentation. For calculating the profiles, nitrogen consumption in the semi-continuous phase was assumed to be zero, as there was no growth of biomass and production of lovastatin does not require nitrogen. Consequently, the change in nitrogen

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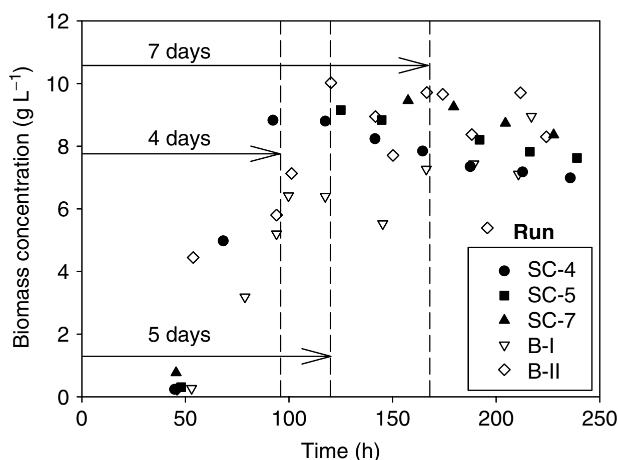

Figure 2. Biomass concentration *versus* fermentation time. Runs labeled SC are for the semi-continuous mode of operation. Vertical lines demarcate the end of the batch phase for runs SC-4 (4 days batch), SC-5 (5 days batch), and SC-7 (7 days batch), respectively. Data for the batch runs B-I and B-II are from for the same fungus, growth medium, and bioreactor[12].

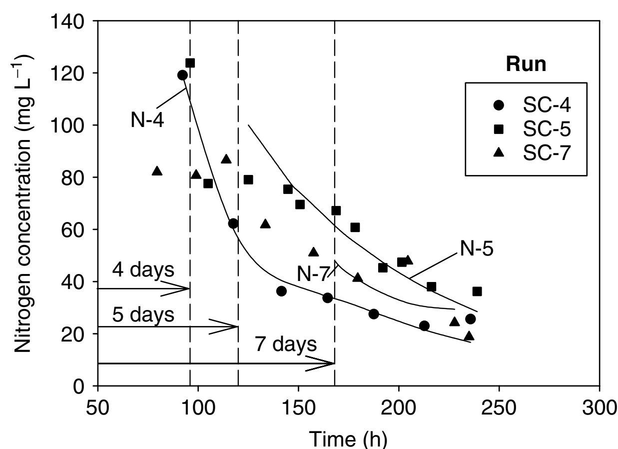

Figure 3. Nitrogen concentration profiles during dilution phase. Lines N4–7 represent concentrations calculated by mass balances for the semi-continuous runs SC-4–7, respectively. Vertical lines demarcate the end of the batch phase for runs SC-4 (4 days batch), SC-5 (5 days batch), and SC-7 (7 days batch), respectively.

concentration was linked directly to nitrogen loss in the effluent stream. The computed profiles are consistent with measured concentration of nitrogen in the broth, confirming the decline in nitrogen concentration to low levels by day 4.

Previously, growth morphology of *A. terreus* had been identified as having an important influence on production of lovastatin.[4,8] Therefore, the fungal growth morphology at various stages of runs SC-4–7 was characterized in terms of pellet diameter and filament ratio (Fig. 4). Figure 4 also includes data that have been reported for runs B-I and B-II, which involved exclusively batch fermentations in the same bioreactor.[12] Clearly, the fed-batch and semi-continuous culture operations did not have any effect on the diameter of fungal pellets and the filament ratio of the pellets in comparison with batch cultures (Fig. 4). The length of the batch phase prior to the start of feeding had no effect on fungal morphology. This was because nitrogen had become limiting by day 4 and growth in all runs had ceased around this time. Comparable values of pellet diameters and filament ratios for the batch and semi-continuous fermentations suggested that any morphology-associated factors would not explain possible differences in lovastatin productivities of these runs, as discussed later in this paper.

In all cases, the measured average fungal pellet diameter at inoculation was $\sim$1500 μm (Fig. 4(b)). As growth occurred up to day 4 in the batch phase, the pellets increased in size to around 2843 ± 18 μm. The pellet diameter then gradually declined to around 2371 ± 212 μm. This was apparently because the pellets were being continuously abraded by the fluid micro-eddies in the broth, but the formation of new biomass (i.e., growth) had ceased by around 100 h when nitrogen had become limiting. Only micro-eddies that are smaller than the dimensions of the pellets are generally capable of abrading a particle and affecting its morphology.[13] Under the conditions used the calculated Kolmogorov length scale of the micro-eddies was approximately 1000 μm, or substantially smaller than the dimensions of the pellets. A similar

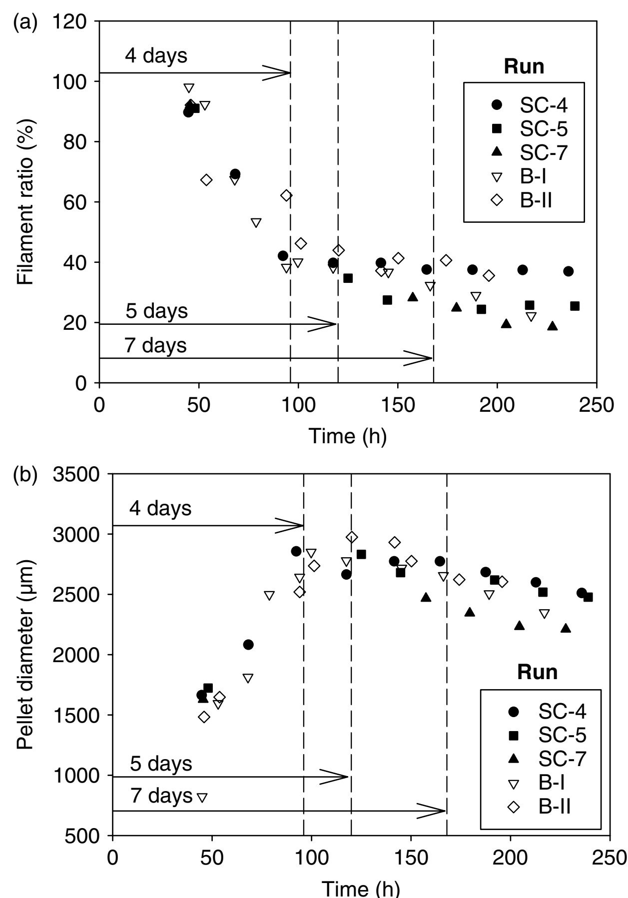

Figure 4. (a) Filament ratio *versus* fermentation time; (b) pellet diameter *versus* fermentation time. SC, semi-continuous; B, batch. Vertical lines demarcate the end of the batch phase for runs SC-4 (4 days batch), SC-5 (5 days batch), and SC-7 (7 days batch), respectively.

micro-eddy length scale of 1200 μm has been estimated for *Aspergillus oryzae* fermentations carried out in stirred tanks.[14] The Kolmogorov length scale l of the micro-eddies was calculated using the following equation:[15]

$$l = \left(\frac{\mu_L}{\rho_L}\right)^{3/4} (gU_G)^{-1/4} \quad (1)$$

where μ_L is the viscosity of the broth, ρ_L is the density of the broth, g is gravitational acceleration, and U_G is the superficial gas velocity in the slurry bubble column reactor.

Rheological behavior of the bulk broth fitted the Ostwald–deWaelle power law model. Figure 5 shows the K- and n-values of the broth at different stages of the fermentation in the various runs. The consistency index K, a measure of the thickness of the broth, was generally much lower in the semi-continuous runs (SC-4–7) in comparison with the batch runs B-I and B-II (Fig. 5(a)) soon after the dilution phase commenced. This was because ongoing dilution with fresh feed caused a preferential washout of the small free hyphae from the bioreactor. Thus, for each of the runs SC-4, SC-5 and SC-7, the K-value dropped substantially soon after the semi-continuous culture operation commenced (Fig. 5(a)). Although the fraction of the biomass that was present in the form of free hyphae was small even before dilution commenced, it contributed substantially to the K-value of the broth.

In batch cultures, although a high proportion of the fungal biomass formed pellets, some hyphae remained in the broth as free filaments or small fluffy clumps. The proportion of pellets to hyphae changed with the progress of the batch fermentation. Nearly 90% of the total biomass existed as pellets at the beginning of all fermentations. Around 120 h of cultivation, when the pellet diameter reached its maximum value, the pelleted morphology represented about 70% of the biomass. The 30% of the biomass that was present as free hyphae was sufficient to cause extensive interactions among pellets by entangling with the external hyphae in the pellets and thus causing the pellets to bridge. This effect of the free hyphae greatly contributed to making the broth highly viscous. Later in the fermentation, a limiting amount of nitrogen prevented further growth of hyphae and actually caused some lysis of the free filaments and a reduction in size of the pellets. Around 200 h, pellet–pellet interactions were reduced sufficiently to greatly reduce the K-value of the broth (Fig. 5(a)). An earlier study of rheology of pelleted broths of *A. terreus*[8] did not recognize the significant contribution of the free hyphal biomass to viscosity of largely pelleted broths, as that study was conducted exclusively in the batch mode of operation. Consequently, there was no washout of free hyphae and their effect on rheology could not be discerned.

In both the batch and semi-continuous cultures, the initial n-values of the broths were similar, as expected

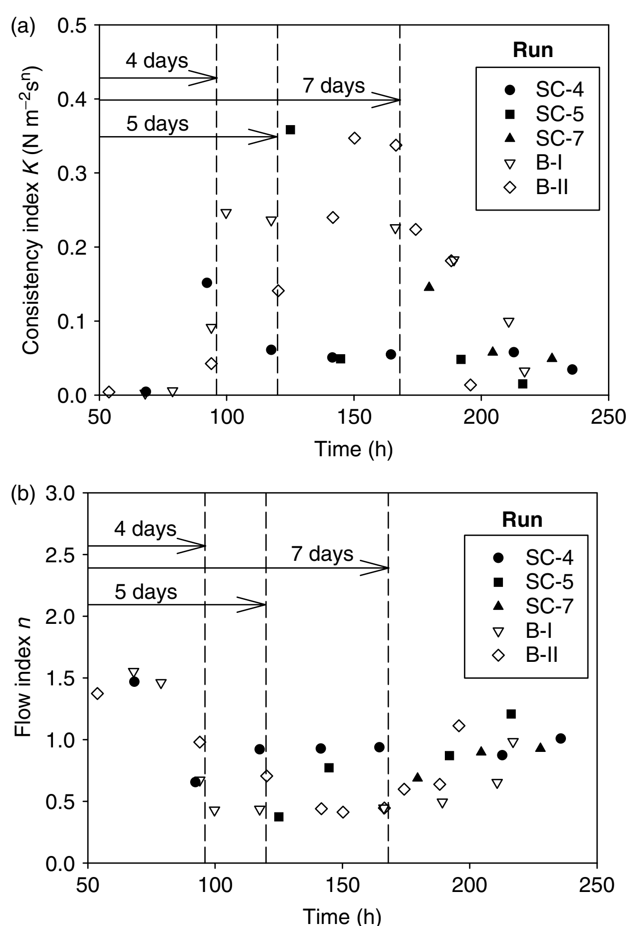

Figure 5. Comparison of rheological parameters in batch (B-I–II) and semi-continuous cultures (SC-4–7). (a) Consistency index; (b) flow index. Vertical lines demarcate the end of the batch phase for runs SC-4 (4 days batch), SC-5 (5 days batch), and SC-7 (7 days batch), respectively.

(Fig. 5(b)) in view of identically prepared inocula. However, soon after the start of dilution and the accompanying preferential loss of the free hyphae, the n-value of the broths of the semi-continuous runs SC-4–7 approached just a little below unity, indicating a slightly shear-thinning fluid (Fig. 5(b)). In contrast, in the batch cultures B-I and B-II the n-values were substantially less than unity between day 4 and day 7 of the culture (Fig. 5(b)), indicating a strongly shear-thinning broth compared with that in the semi-continuous runs. Towards the end of the batch runs, the n-values of the broth once again became comparable to those of the semi-continuous runs because of the above explained lysis of free hyphae later in batch operation when nitrogen had become limiting.

Concentration of lovastatin in the broth at various times during the fermentations is shown in Fig. 6. As expected, lovastatin concentration in the batch runs (i.e., B-I and B-II) are comparable with the concentrations in the semi-continuous runs SC-4–7 until the dilution phase commences in the semi-continuous runs. Once dilution starts, the lovastatin concentration in the broth of semi-continuous runs becomes substantially less than in the broths of the

batch runs, as was intended by the design of the feeding strategy. The reduced prevailing concentration of lovastatin in runs SC-4–7 actually improved lovastatin productivity, as was intended. This is discussed next.

The actual rate of generation of lovastatin in the bioreactor is shown in Fig. 7 for various times during the fermentation. The generation rate was calculated with the following mass balance on lovastatin:

$$\frac{dC_{lov}}{dt} = r_{lov} - DC_{lov} \qquad (2)$$

where C_{lov} is the concentration of lovastatin in the broth at time t, r_{lov} is the rate of production of lovastatin, and D is the dilution rate. For calculating r_{lov}, smooth curves or straight lines were plotted through the relevant data in Fig. 6. The left-hand-side of Eqn (2) was read as the slope of the tangent at any point on the graph. The corresponding value of C_{lov} was read directly from the graph (Fig. 6). The dilution rate (D) was always $0.26\,d^{-1}$, as noted earlier.

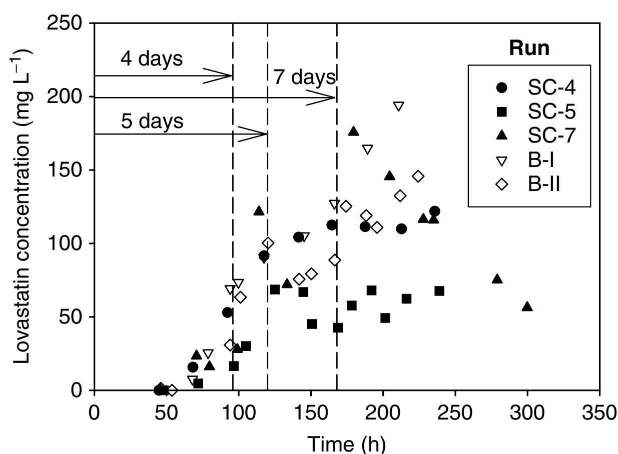

Figure 6. Effect of dilution on lovastatin concentration. SC, semi-continuous; B, batch (no dilution). Vertical lines demarcate the end of the batch phase for runs SC-4 (4 days batch), SC-5 (5 days batch), and SC-7 (7 days batch), respectively.

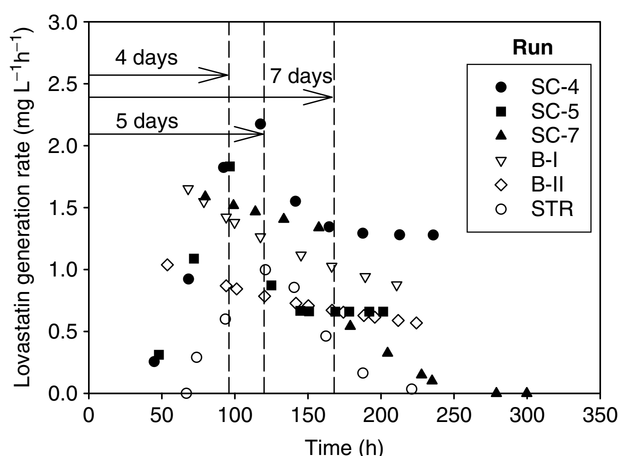

Figure 7. Variation in lovastatin generation rate with culture time. Data labeled STR are for a stirred-tank fermentation conducted with the same fungus using the same medium.[7] Vertical lines demarcate the end of the batch phase for runs SC-4 (4 days batch), SC 5 (5 days batch), and SC-7 (7 days batch), respectively.

Clearly, prior to dilution commencing, the generation rates of lovastatin in the semi-continuous and batch runs are quite similar (Fig. 7). Once dilution commences, substantially higher generation rate is seen in run SC-4 compared with the batch runs B-I and B-II (Fig. 7). The semi-continuous runs SC-5 and SC-7 are essentially identical to the batch runs B-I and B-II in the generation rates of lovastatin, as dilution commenced late in these runs compared with the best semi-continuous run SC-4. This indicated that a batch phase of 4 days was optimal for attaining a high generation rate of lovastatin in subsequent semi-continuous production. The SC-4 feeding strategy increased lovastatin generation rate by >50% compared with the batch runs B-I and B-II, as seen in Fig. 7.

Because of a lower shear environment, the bubble column slurry reactor used here performed distinctly better than the same fermentation conducted in a stirred-tank bioreactor.[7] This is shown in Fig. 7, where the lovastatin generation rate values for the stirred-tank culture (i.e., STR in Fig. 7) are low compared with the semi-continuous runs (SC-4–7) and the batch runs (B-I, B-II) carried out in the slurry bubble column. The stirred-tank fermentation STR (Fig. 7) had been carried out using exactly the same environmental conditions, fungal strain, and medium as used in the present work. The only difference was that the stirred bioreactor had an average shear rate of $\sim 70\,s^{-1}$ at the 300 rpm agitation speed used.[7] Under these conditions the average maximum value of the pellet diameter in the stirred bioreactor[7] was 2500 μm *versus* an average maximum value of 2900 μm in the bubble column slurry reactor (Fig. 4). The filament ratio values in both types of bioreactors at 100 h were similar at 40%.

CONCLUDING REMARKS

In production of the secondary metabolite lovastatin by *A. terreus*, a preferred feeding strategy is to use a two-stage fermentation with retention of the biomass pellets in a slurry bubble column bioreactor. The preferred feeding strategy involves an initial batch phase lasting a minimum of 4 days and a semi-continuous culture phase. The feeding in semi-continuous operation serves only to sustain the biomass without producing growth. This is achieved by nitrogen limitation in the feed medium. The preferred feeding strategy enhanced lovastatin production rate by >50% (i.e., SC-4) compared with batch culture in a slurry bubble column because dilution with fresh non-growth-promoting medium reduced self-inhibition of lovastatin synthesis. Use of a bubble column slurry reactor that did not cause excessive disruption of fungal pellets was shown to be superior to fermentation in a conventional stirred tank. The feeding strategy identified here is potentially applicable to production of various other fungal metabolites whose production is non-growth associated and favored by a pelleted growth morphology.

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