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Lovastatin production by *Aspergillus terreus* in a two-staged feeding operation

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Abstract

BACKGROUND: Lovastatin is known to inhibit its own synthesis in the fungus *Aspergillus terreus*. Therefore, the use of a fermentation strategy that continuously removes some of the lovastatin produced from the bioreactor can enhance its productivity. This paper reports on the effects of dilution rate and the composition of the feed medium on lovastatin production by *A. terreus*.

RESULTS: The feeding strategy consisted of an initial batch/fed-batch phase and a semi-continuous culture phase in which the pelleted biomass was retained inside a slurry bubble column. A nitrogen-free medium was fed at various fixed dilution rates in the semi-continuous phase. In experiments that were designed to assess the effects of the composition of the medium, the dilution rate was held at 0.42 d^{-1}, but different feed media were used in separate runs. The best two-staged production strategy was shown to consist of a 96 h batch/fed-batch phase that used a nutritionally complete medium. This was followed by a semi-continuous operation using a medium that was free of both nitrogen and carbon sources.

CONCLUSION: Semi-continuous operation enhanced productivity of lovastatin by 315% compared with a conventional batch operation. The optimal dilution rate in semi-continuous operation was about 0.42 d^{-1}.

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

NOMENCLATURE

C_{lov}	Concentration of lovastatin at time t (mg L^{-1})
D	Dilution rate (h^{-1}) in Eqn (1)
g	Gravitational acceleration (m s^{-2})
K	Consistency index of broth (N m^{-2} s^n)
M-A	Fermentation medium A
M-B	Fermentation medium B
M-C	Fermentation medium C
M-CII	Duplicate run with the medium M-C
n	Flow behavior index of broth
r_{lov}	Rate of production of lovastatin (mg L^{-1} h^{-1})
t	Time (h)

INTRODUCTION

Lovastatin, a secondary metabolite of the filamentous fungus *Aspergillus terreus*, is used to reduce blood cholesterol. Both stirred tank and bubble column bioreactors can be used to produce lovastatin.[1–5] Lovastatin biosynthesis depends on fungal morphology and oxygen concentration.[3,5,6] Previous studies have reported an increased lovastatin production when *A. terreus* was grown as pellets instead of in freely dispersed filamentous growth. Fungal pellets consist of a central compact core region and a peripheral filamentous or hairy region.[7,8] Lovastatin production in pellets takes place in the external filamentous area,[5] because the core region is formed primarily by lysed cells.[9] The high viscosity of fungal fermentation broth produces a number of problems. One of these is the decrease in mass transport from the gas to the liquid phase as a result of bubble coalescence. In stirred tank bioreactors, high agitation speeds that increase gas–liquid mass transfer disintegrate pellets to reduce production of lovastatin.[1,2,5,7] Several studies propose that the interaction between pellets and eddies is the most probable mechanism of pellet disruption.[10,11] Use of bubble column bioreactors reduces pellet disintegration and can provide sufficient oxygen mass transfer for this fermentation.[6]

Lovastatin synthesis is self inhibitory[12] and this limits culture productivity in batch mode of fermentation. A continuous dilution of the culture bulk reduces lovastatin concentration and, therefore, the inhibitory effect, and increases total production of lovastatin.[13–15]

Although the production of lovastatin by *A. terreus* in batch fermentations has been studied extensively, fed-batch and repeated fed-batch operations are known

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to enhance productivity of lovastatin compared with batch culture.[13,14] Earlier studies have not given any information regarding the effect of medium composition in fed-batch production of lovastatin. A two-stage semi-continuous mode of fermentation has been used to improve productivity.[15] This two-stage semi-continuous operation consisted of an initial batch/fed-batch phase that built up the biomass. This was followed by a semi-continuous culture phase at a fixed dilution rate in a slurry bubble column that retained the biomass pellets within the reactor. How the dilution rate might affect productivity in a two-stage semi-continuous operation is unknown.

This work reports on the effects of changes in dilution rate and the composition of the feed medium on lovastatin production by two-stage semi-continuous culture of *A. terreus*. Dilution rate in the semi-continuous phase was varied between 0.13 and 0.50 d^{-1} using a feed medium that was free of nitrogen. During studies with media of different compositions, the dilution rate in the semi-continuous phase remained at 0.42 d^{-1}, but, in separate runs, the feed medium varied to span a nutritionally complete medium, a nitrogen-free medium and a medium that was free of both nitrogen and carbon sources.

MATERIALS AND METHODS

Microorganism, inoculation and growth conditions

Aspergillus terreus ATCC 20 542 was obtained from the American Type Culture Collection. The fungus was maintained in Petri dishes of potato dextrose agar (PDA). The bioreactor was inoculated with pellets obtained by germination from spores suspended in shake flasks, as explained previously.[15]

Fermentations lasted for around 10 days. The culture medium contained lactose as the carbon source and soybean meal as the 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, per liter: 100 mg $Na_2B_4O_7.10H_2O$, 50 mg $MnCl_2.4H_2O$, 50 mg $Na_2MoO_4.2H_2O$, and 250 mg $CuSO_4.5H_2O$. 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 (Fig. 1). The diameter of the reactor vessel was 0.155 m and the culture high was 0.9 m. Gas was sparged through a perforated plate (150 holes of 1.5 mm diameter) located at the base of the reactor. Other operational details of the reactor have been published.[15]

During the semi-continuous culture phase, fresh medium was fed using a diaphragm pump through a port located on top of the reactor. Three different feed media were used in separate experiments. Medium M-A comprised of 1.79 g L^{-1} soybean meal as the nitrogen source, 37.93 g L^{-1} lactose as the carbon source, and above specified levels of various mineral salts. Medium M-B contained mineral salts as specified above, 114.26 g L^{-1} lactose and no nitrogen source. Medium M-C consisted of the above specified mineral salts and no sources of carbon and nitrogen. The dilution rate values tested were 0.13, 0.26, 0.42 and 0.50 d^{-1}. The feed pump was calibrated before each experiment. The withdrawal of

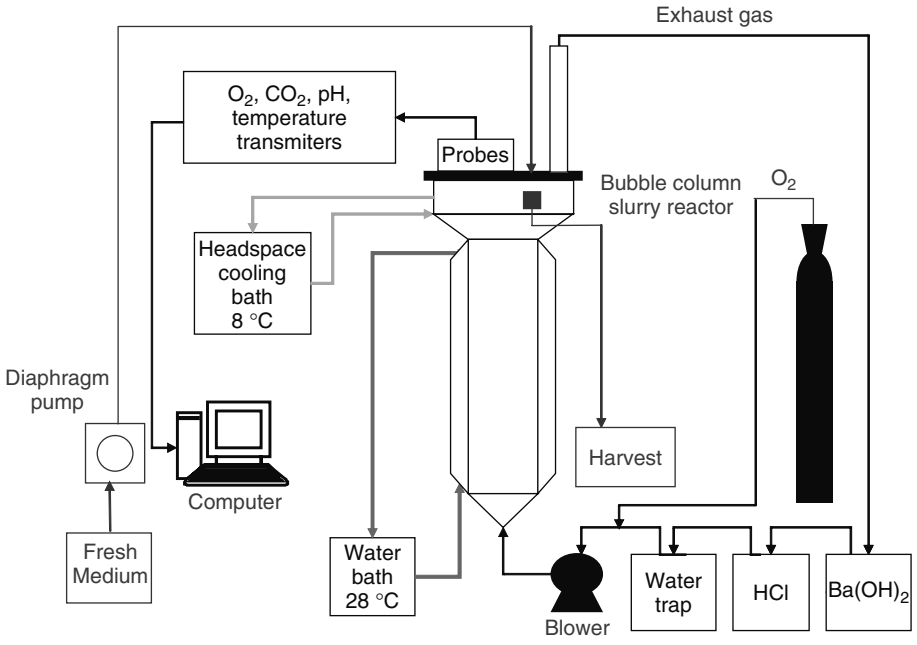

Figure 1. Bioreactor setup.

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/fed-batch phase 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. The batch phase lasted 96 h. This time included 48 h in the shake flask stage that was used for inoculation of the reactor. After the batch period, approximately 2.4 L of medium was added over the course of 12 h in a fed-batch phase until the broth level reached the harvest port. Semi-continuous culture operation with pellet retention commenced as soon as the broth level reached the harvest port.

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 by Casas López *et al*.[5]

Morphological measurements

The fungal pellet morphology was characterized using image analysis.[16] 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). Prior to imaging, each sample of the fermentation broth was processed by decanting 10 mL of sample and then washing twice with 20 mL distilled water. Within a sample, 100 objects were analyzed for each determination.

The changes in pellet morphology were quantified using the following two measures: (1) the pellet diameter, defined as the diameter corresponding to a circular area equivalent to the total pellet projected area; and (2) the 'filament ratio', which is the ratio between the area of the peripheral 'hairy surface' and the total area of the pellet. These two measures provided a direct indication of the pellet size and the proportion of productive biomass present.

Analytical methods

The biomass was determined as dry weight and the lovastatin was measured in its β-hydroxyacid form by high-performance liquid chromatography (HPLC) of the biomass-free filtered broth. The methods used to measure the biomass and lovastatin concentrations have been described.[15]

Total nitrogen concentration in the culture samples was measured by a total organic carbon analyzer TOC-VCPN (Shimadzu Corp., Kyoto, Japan) equipped with a total nitrogen unit (Shimadzu). The sample was oxidized in the presence of a catalyst by combustion at 720 °C and luminescence of the gas produced was measured. The sample had been previously filtered with a 0.45 µm Millipore membrane filter and diluted with Milli-Q deionized water.

RESULTS AND DISCUSSION

Effects of dilution rate

The feed medium used during the semi-continuous phase of fermentation was always the medium M-B for studies discussed in this section.

Figure 2 shows the variation of the biomass concentration with fermentation time for the four studied dilution rates. All fermentations displayed similar values of growth rates and attained similar biomass levels during the first 96 h of batch operation (no dilution), indicating highly reproducible growth characteristics. After 96 h of fermentation, semi-continuous operation started at the specified dilution rate values (Fig. 2). The biomass concentration declined slightly with time during the semi-continuous operation. The rate of concentration decline increased with increasing dilution rate (Fig. 2). The decrease in biomass concentration during dilution was due to washout of a small fraction of the biomass that existed in non-pelleted free mycelial form.

Biomass did not grow during the semi-continuous phase because the nitrogen level in the medium had been depleted to growth-limiting levels by the end of the batch phase and the medium fed during the semi-continuous phase did not contain any nitrogen. Figure 3 shows the nitrogen concentration at various stages of the fermentations. Low levels of nitrogen are obvious after the first 96 h of batch fermentation. All fermentations were provided with a high excess of the carbon source; consequently, there was a negligible depletion in the concentration of the carbon source during the fermentation (data not shown).

In earlier studies,[8,12] growth morphology of *A. terreus* has been identified as having an important

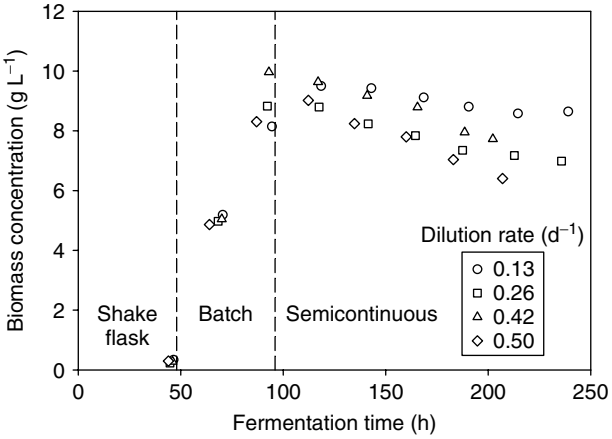

Figure 2. Biomass concentration *versus* fermentation time at different dilution rates.

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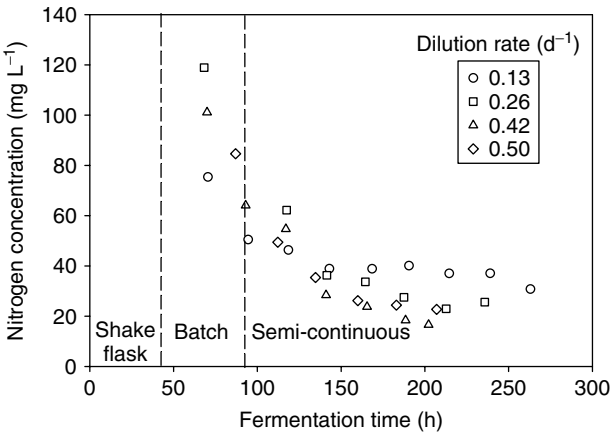

Figure 3. Residual nitrogen concentration *versus* fermentation time at different dilution rates.

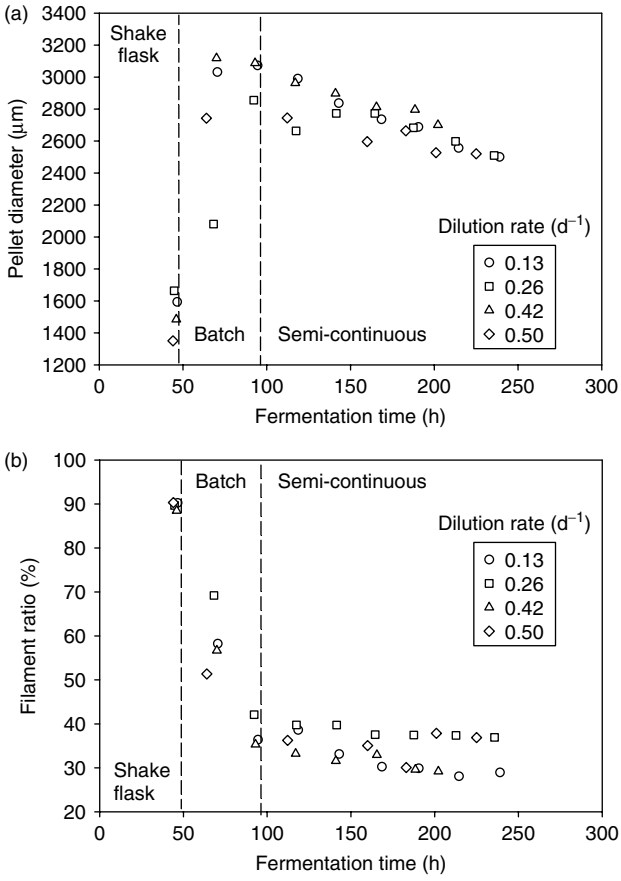

Figure 4. Pellet diameter (a) and filament ratio (b) *versus* fermentation time at various dilution rates.

influence on the production of lovastatin. Therefore, there is a need to differentiate between the effects of fungal morphology on lovastatin production from the influence of the dilution rate. Growth morphology was characterized by measurements of the pellet diameter (Fig. 4(a)) and the filament ratio (Fig. 4(b)). In all the fermentations, the average diameter of the pellets used to inoculate the bubble column bioreactor was roughly 1500 μm and the pellet size increased to around 3000 μm during fungal growth in the batch phase (Fig. 4(a)). The pellet diameter gradually declined during the semi-continuous phase and reached a value of 2500 μm after 200 h of fermentation (Fig. 4(a)). The decrease in pellet diameter was due to erosion of the pellets' surfaces by the fluid microeddies in the broth in the absence of any new biomass being formed, i.e., in the absence of growth.

The influence of turbulence in the fluid on morphology of fungal pellets is widely recognized.[5,7,17] Microeddies that are smaller than the dimensions of the pellets are generally capable of abrading a particle because they produce hydrodynamic pressure fluctuations on the opposite sides of pellets.[17] Under the conditions used in these fermentations, the Kolmogorov length scale of the microeddies was approximately 1000 μm, or substantially smaller than the dimensions of the pellets[15] and, therefore, fluid eddies were concluded to reduce the size of the pellets.

Pellet size actually increased during the batch phase because biomass generation by growth exceeded the biomass loss due to surface erosion; however, in the semi-continuous (i.e., after 96 h of fermentation) the nitrogen concentration in the broth was insufficient to allow continued growth (Fig. 3). Filament ratio declined with time from an initial value of around 90% to approximately 35% by the end of the batch phase (Fig. 4(b)). Later in the fermentation, the filament ratio remained unchanged.

Dilution rate did not have a substantial effect on either the pellet diameter or filament ratio. The pellet diameter and the filament ratio profiles in fermentations with the two feeding stages were similar to those reported[6] in batch fermentations in a bubble column bioreactor under culture conditions that were otherwise identical to those used in the present work; therefore, any changes in lovastatin production relative to the earlier study[6] were concluded to be due to dilution, which reduced the prevailing concentration of lovastatin. This in turn reduced product inhibition of the fermentation.

Rheological properties of the culture broth, characterized in terms of the consistency index (K) and flow behavior index (n), depended on dilution rate, as shown in Fig. 5. During batch operation, the K values increased with culture time in all the fermentations, whereas the n values decreased. Increase in K values is associated with increasing 'thickness' of the fluid because of increasing biomass concentration, pellet diameter and the fraction of free hyphae (Fig. 2).[15] The decline in n values suggests an increasingly shear-thinning rheology as the biomass concentration increased. During semi-continuous operations the K values declined with time (Fig. 5(a)) as the non-pelleted fraction of the biomass was washed out, confirming earlier observations.[15] At a low dilution rate value of 0.13 d^{-1}, the variation in K with fermentation time was analogous to that described for batch cultures[6] because of a slow washout of the non-pelleted biomass. Washout of non-pelleted biomass increased with increasing dilution rate. During semi-continuous operation, at dilution rates of 0.42 d^{-1} and 0.50 d^{-1} the n values increased with time (Fig. 5(b))

as the broth contained less biomass in the form of free filaments. At the lowest dilution rate, n continued to decrease for 24 h after dilution commenced and then increased because of some lysis of free filaments caused by aging of the biomass.

Figure 6 shows lovastatin concentration *versus* fermentation time. Lovastatin concentration increased with fermentation time in the batch phase as biomass grew. Lovastatin concentration continued to increase slightly in the semi-continuous phase even though biomass growth had ceased after 96 h of fermentation at a dilution rate of $0.13\,d^{-1}$ and $0.26\,d^{-1}$ (Fig. 6). Lovastatin concentration did not change with fermentation time at a dilution rate value of $0.42\,d^{-1}$ (Fig. 6). At a dilution rate value of $0.50\,d^{-1}$, the removal rate of lovastatin exceeded its rate of production and hence there was a decline in concentration with fermentation time (Fig. 6).

Figure 7 shows the rate of generation of lovastatin in the bioreactor for different fermentations. The generation rate was calculated with the following mass balance on lovastatin:

$$\frac{dC_{lov}}{dt} = r_{lov} - DC_{lov} \quad (1)$$

In Eqn (1), 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

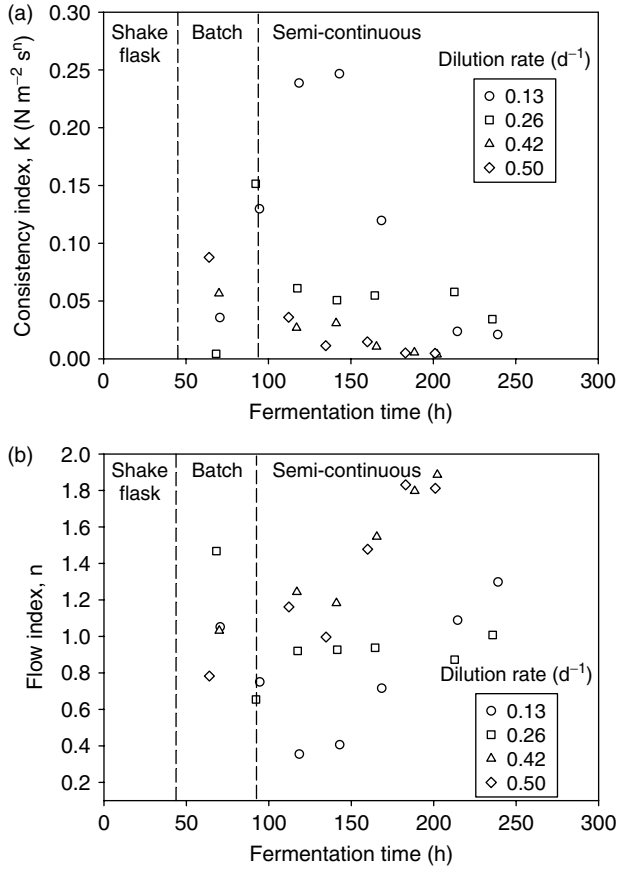

Figure 5. Broth consistency index (a) and flow behavior index (b) *versus* fermentation time at various dilution rates.

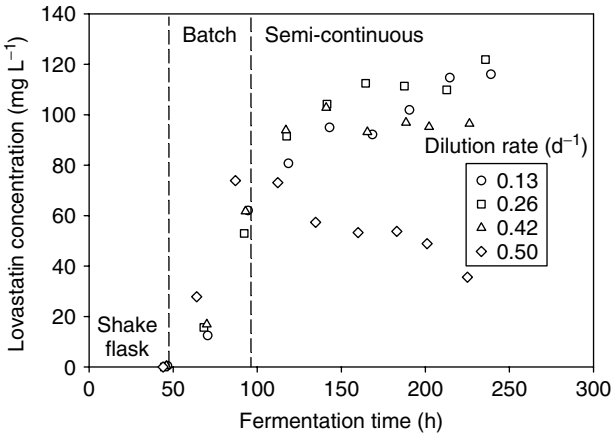

Figure 6. Lovastatin concentration *versus* fermentation time at various dilution rates.

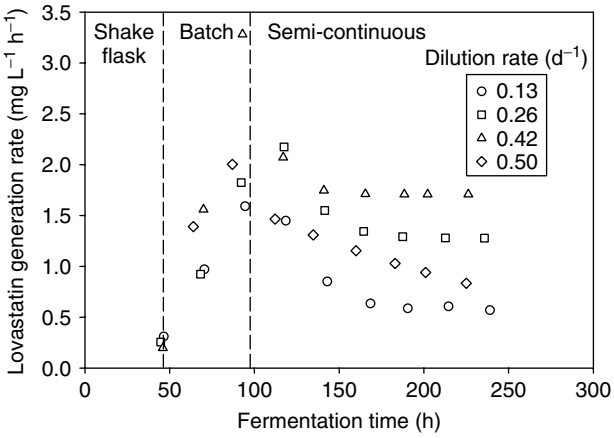

Figure 7. Lovastatin generation rate *versus* fermentation time at various dilution rates.

through the relevant data. The left-hand side of Eqn (1) 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.

The rate of generation of lovastatin increased with time during the batch phase in all the fermentations (Fig. 7). Once the semi-continuous operation had started, lovastatin generation rate declined rapidly with time at dilution rates of 0.13 and $0.50\,d^{-1}$ (Fig. 7), but it did not vary with time at dilution rates of 0.26 and $0.42\,d^{-1}$ (Fig. 7). Lower (i.e., $D = 0.13\,d^{-1}$) and higher (i.e., $D = 0.50\,d^{-1}$) dilution rates were not satisfactory for maintaining a high stable rate of generation of lovastatin (Fig. 7). As discussed above, there was a generally low filament ratio after 96 h of fermentation (Fig. 4(b)) because of a loss of some 'productive' biomass (Figs 2 and 4(b)) and this explained the reduced generation rate of lovastatin in the semi-continuous phase.

If we compare the results in Fig. 7 with data reported for the batch mode of operation,[6] a large effect is seen of the dilution rate on the lovastatin generation rate. Dilution was not accompanied by any significant changes in pellet morphology that could explain the differences in lovastatin production in batch

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and semi-continuous fermentations. Consequently, changes in lovastatin production were attributed to culture dilution.

The best lovastatin production was achieved at a dilution rate of $0.42\,d^{-1}$. The average lovastatin generation rate was slightly lower for semi-continuous fermentation at a dilution rate of $0.13\,d^{-1}$ than for batch fermentations and slightly higher at a dilution rate of $0.50\,d^{-1}$.

Effects of medium composition

The effects of media composition on fungal growth and lovastatin production in the semi-continuous phase were studied using three different media, i.e., media M-A, M-B and M-C. Medium M-A was a complete medium that contained carbon (lactose) and nitrogen (soybean meal) sources, salts, and necessary vitamins as specified in 'Materials and methods'.

The possibility of extending the duration of fermentation by feeding this complete medium was studied. If the aging of the fungus and a decrease in lovastatin production are due to a limitation of nitrogen, the semi-continuous addition of the nitrogen source should postpone the cessation in lovastatin production to a later time. Medium M-B had the carbon source and all the other relevant nutrients, but no nitrogen source because nitrogen is not required for synthesis of lovastatin. Medium M-C lacked carbon and nitrogen sources, but it contained the necessary salts and vitamins. Usually, secondary metabolism requires a minimal concentration of nutrients and, using medium M-C, we wanted to see if lovastatin production would continue to occur under low nutrient concentrations. In all cases, the concentrations of salts and biotin were the same as in the batch phase experiments. The batch phase medium was always the complete medium detailed in a previous section. In all cases, the semi-continuous operation started at 96 h, and the dilution rate was held constant at approximately $0.42\,d^{-1}$.

Figure 8 shows biomass concentration *versus* time data for various fermentations. The medium denoted as M-CII (Fig. 8) was simply a duplicate fermentation conducted with the medium M-C. There was no biomass growth after 96 h when semi-continuous operation commenced for the two media that did not contain nitrogen (Fig. 8) because the nitrogen from the batch phase had been depleted. In these fermentations there was also a slight decline in biomass concentration with time (Fig. 8) as a consequence of washout of the non-pelleted fraction of the biomass. The growth profiles of the runs M-C and M-CII matched closely (Fig. 8), confirming a high level of reproducibility of data. The biomass growth data for the runs M-C and M-B were virtually identical (Fig. 8), indicating that nitrogen and carbon were not necessary for sustaining the non-growing biomass.

Conversely, biomass continued growing until 200 h in the semi-continuous phase after feeding with the complete medium M-A had commenced (Fig. 8). The

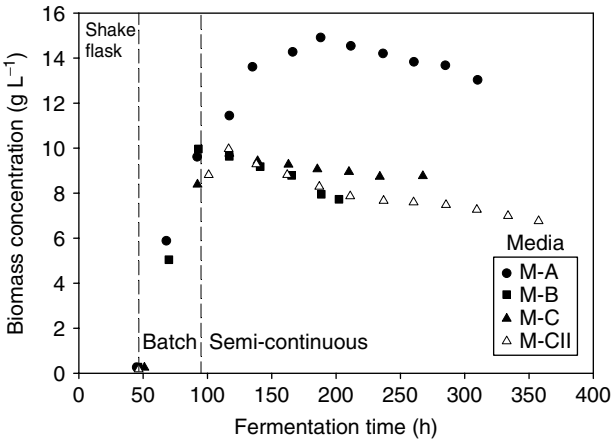

Figure 8. Biomass concentration *versus* fermentation time in various media.

biomass concentration did not change after 200 h of fermentation because of hyphal washout. This indicated that a high concentration of nitrogen could not prevent the aging of the retained fungal pellets beyond about 200 h.

Figure 9 shows the nitrogen concentration data. In batch operation, the nitrogen levels in all the fermentations declined rapidly with time at comparable rates because of consumption by the growing biomass (Fig. 9). Nitrogen concentration rapidly decreased to low levels once the dilution commenced in nitrogen-free media (i.e., media M-B, M-C and M-CII) (Fig. 9). The results for nitrogen-containing media M-A are quite different (Fig. 9). For this medium, nitrogen concentration continued to decrease for up to ~200 h in the semi-continuous phase (Fig. 9) because of consumption by growing biomass (Fig. 8). Afterwards, the nitrogen concentration in the broth started to increase (Fig. 9), indicating a reduced consumption rate of nitrogen by the biomass. This decline in nitrogen consumption was associated with the aging of biomass pellets, as mentioned above. Clearly, aging of the pellets that are retained in the bioreactor cannot be prevented forever even if the culture is fed with a complete medium and the essential nutrients are available in abundance. The carbon level in the medium M-A was in high excess and was negligibly affected during the fermentation.

Regarding the pellet diameter and filament ratio, there were no significant differences between runs and the general behavior seen was consistent with that discussed above, irrespective of the medium used.

During semi-continuous operation, the lovastatin concentrations attained in the broth (Fig. 10) of the complete medium M-A were substantially lower than the concentration obtained in fermentations with nitrogen-free media (i.e., M-B, M-C and M-CII). Furthermore, the rates of generation of lovastatin (Fig. 11) were substantially lower in the complete medium (i.e., M-A) when compared with the corresponding values in nitrogen-deficient media M-C and M-CII.

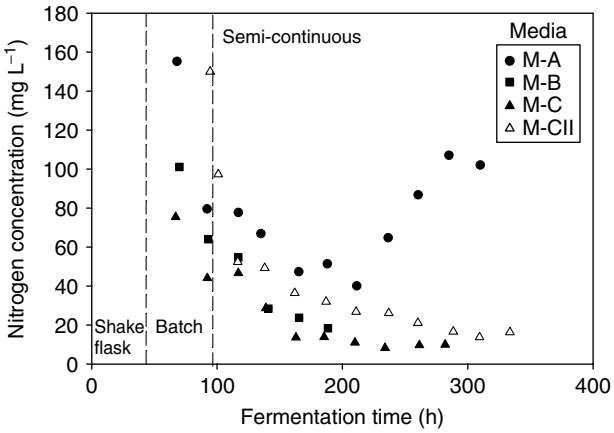

Figure 9. Residual nitrogen concentration *versus* fermentation time in various media.

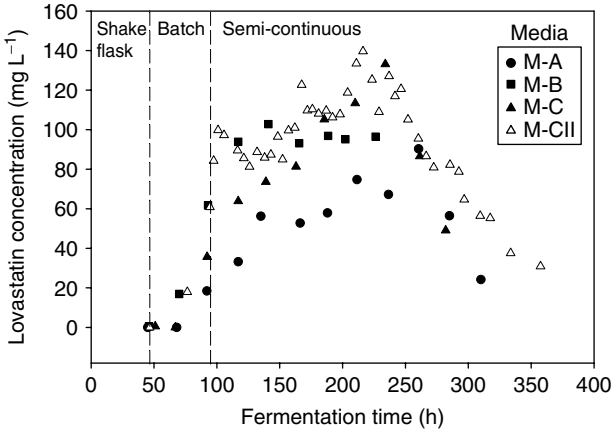

Figure 10. Lovastatin concentration *versus* fermentation time in various media.

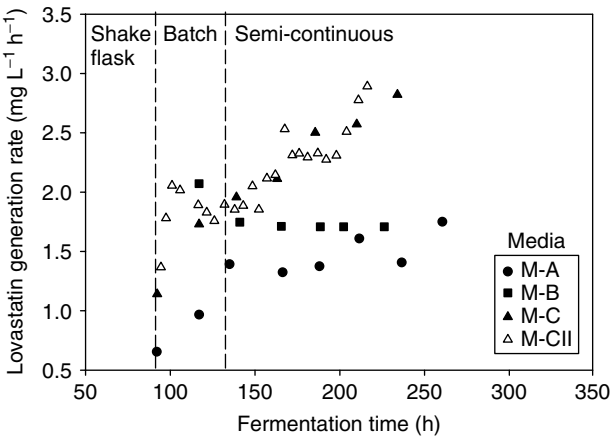

Figure 11. Lovastatin generation rate *versus* fermentation time in various media.

These results demonstrate that it is not necessary to maintain a high concentration of nutrients and the positive effect of lovastatin dilution is sufficient to increase lovastatin production. Furthermore, in nitrogen-containing media, the rate of production of lovastatin was lower than in media that do not contain nitrogen.

Fed-batch fermentations have been suggested for increasing the productivity of lovastatin relative to batch operation.[13,14] Fed-batch operations certainly dilute the broth to reduce concentration of lovastatin and therefore reduce the self-inhibition of its synthesis,[8] but they do not do so as effectively as the semi-continuous operation does. Also, the fed-batch production strategies proposed previously all relied on nitrogen-containing media for the feeding and, consequently, they were less favorable for producing lovastatin in comparison with the nitrogen-free semi-continuous operation.

Optimal combination of medium and dilution rate

The combination of medium M-C and a dilution rate of $0.42\,d^{-1}$ in the semi-continuous phase increased lovastatin production by 93% in comparison with conventional batch fermentation in a bubble column[6] and by 315% compared with a batch stirred tank culture.[5] These enhancements were substantial compared with the previously reported[11] increase of 50% with a medium that contained a carbon source, but no nitrogen, and was fed at a dilution rate of $0.26\,d^{-1}$.

CONCLUSIONS

A nitrogen-free feed medium that contained only the mineral salts and biotin was shown to be best suited for semi-continuous operation. The optimal lovastatin production strategy identified in this paper consisted of using a batch/fed-batch phase for the first 96 h, followed by a semi-continuous phase at a dilution rate of about $0.42\,d^{-1}$ for a further 140 h. This fermentation strategy combined the benefits of using low-shear bubble column bioreactors to obtain the preferred pelleted morphology and a reduced self-inhibition of lovastatin production. The two-stage feeding strategy used here increased lovastatin production by 315% compared with the traditional batch fermentations in stirred tank bioreactors.

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REFERENCES

1 Cui YQ, van der Lans RGJM and Luyben KCAM, Effect of dissolved oxygen tension and mechanical forces on fungal morphology in submerged fermentations. *Biotechnol Bioeng* **57**:409–419 (1998).

2 Lai LST, Kuo GM and Tsai SY, Influence of increased dissolved oxygen tensions by agitation on secondary metabolite production by a mutant of *Aspergillus terreus* in a 5L fermentor. *J Chin Inst Chem. Eng* **32**:135–142 (2001).

3 Lai LST, Tsai SY and Wang TC, Application of oxygen vectors to *Aspergillus terreus* cultivation. *J Biosci Bioeng* **5**:453–459 (2002).

4 Casas López JL, Sánchez Pérez JA, Fernández Sevilla JM, Acién Fernández FG, Molina Grima E and Chisti Y, Fermentation optimization for the production of lovastatin by *Aspergillus terreus*: use of the response surface methodology. *J Chem Technol Biotechnol* **79**:1119–1126 (2004).
5 Casas López JL, Sánchez Pérez JA, Fernández Sevilla JM, Rodríguez Porcel EM and Chisti Y, Pellet morphology, culture rheology and lovastatin production in cultures of *Aspergillus terreus*. *J Biotechnol* **116**:61–77 (2005).
6 Rodríguez Porcel EM, Casas López JL, Sánchez Pérez JA, Fernández Sevilla JM, García Sánchez JL and Chisti Y, *Aspergillus terreus* broth rheology, oxygen transfer and lovastatin production in a gas-agitated slurry reactor. *Ind Eng Chem Res* **45**:4837–4843 (2006).
7 Cui YQ, van der Lans RGJM and Luyben KCAM, Effect of agitation intensities on fungal morphology of submerged fermentation. *Biotechnol Bioeng* **55**:715–726 (1997).
8 Rodríguez Porcel EM, Casas López JL, Sánchez Pérez JA, Fernández Sevilla JM and Chisti Y, Effects of pellet morphology on broth rheology in fermentations of *Aspergillus terreus*. *Biochem Eng J* **26**:139–144 (2005).
9 Clark, DS, Submerged citric acid fermentation of ferrocyanide-treated beet molasses: morphology of pellets of *Aspergillus niger*. *Can J Microbiol* **8**:133–136 (1962).
10 Ayazi Shamlou P, Makagiansar HY, Lilly MD and Thomas CR. Turbulent breakage of filamentous microorganisms in submerge culture in mechanically stirred bioreactors. *Chem Eng Sci* **49**:2621–2631 (1994).
11 Van Suijdam C and Metz B. Fungal pellet breakup as a function of shear in a fermenter. *J Ferment Technol* **59**:329–333 (1981).
12 Casas López JL, Rodríguez Porcel EM, Vilches Ferrón MA, Sánchez Pérez JA, Fernández Sevilla JM and Chisti Y, Lovastatin inhibits its own synthesis in *Aspergillus terreus*. *J Ind Microbiol Biotechnol* **31**:48–50 (2004).
13 Novak N, Gerdin S and Berovic M, Increased lovastatin formation by *Aspergillus terreus* using repeated fed-batch process. *Biotechnol Lett* **19**:947–948 (1997).
14 Kumar MS, Jana SK, Senthil V, Shashanka V, Kumar SV and Sadhukhan AK, Repeated fed-batch process for improving lovastatin production. *Process Biochem* **36**:363–368 (2000).
15 Rodríguez Porcel EM, Casas López JL, Sánchez Pérez JA and Chisti Y, Enhanced production of lovastatin in a bubble column by *Aspergillus terreus* using a two-stage feeding strategy. *J Chem Technol Biotechnol* **82**:58–64 (2007).
16 Paul GC and Thomas CR, Characterisation of mycelial morphology using image analysis. *Adv Biochem Eng* **60**:1–59 (1998).
17 Chisti Y, Shear sensitivity, in *Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparation*, Vol. 5, ed. by Flickinger MC and Drew SW. Wiley, New York, pp. 2379–2406 (1999).