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Pellet morphology, culture rheology and lovastatin production in cultures of *Aspergillus terreus*

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Abstract

Pellet growth of *Aspergillus terreus* ATCC 20542 in submerged batch fermentations in stirred bioreactors was used to examine the effects of agitation (impeller tip speed u_t of 1.01–2.71 m s^{-1}) and aeration regimens (air or an oxygen-enriched mixture containing 80% oxygen and 20% nitrogen by volume) on the fungal pellet morphology, broth rheology and lovastatin production. The agitation speed and aeration methods used did not affect the biomass production profiles, but significantly influenced pellet morphology, broth rheology and the lovastatin titers. Pellets of ~1200 μm initial diameter were reduced to a final stable size of ~900 μm when the agitation intensity was ≥600 rpm ($u_t \geq 2.03$ m s^{-1}). A stable pellet diameter of ~2500 μm could be attained in less intensely agitated cultures. These large fluffy pellets produced high lovastatin titers when aerated with oxygen-enriched gas but not with air. Much smaller pellets obtained under highly agitated conditions did not attain high lovastatin productivity even in an oxygen-enriched atmosphere. This suggests that both an upper limit on agitation intensity and a high level of dissolved oxygen are essential for attaining high titers of lovastatin. Pellet size in the bioreactor correlated equally well with the specific energy dissipation rate and the energy dissipation circulation function. The latter took into account the frequency of passage of the pellets through the high shear regions of the impellers. Pellets that gave high lovastatin titers produced highly shear thinning cultivation broths.

Keywords: *Aspergillus terreus*; Lovastatin; Fungal morphology; Broth rheology; Pelleted growth

1. Introduction

Submerged cultures of filamentous fungi are widely used to produce commercially important metabolites including many antibiotics and the cholesterol lowering drugs (statins) such as lovastatin ($C_{24}H_{36}O_5$,

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Nomenclature

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A	parameter defined by Eq. (18)
c	constant in Eq. (2)
D	impeller diameter (m)
D_P	pellet diameter (μm)
EDCF	energy dissipation/circulation function defined by Eq. (1) ($W\,m^{-3}\,s^{-1}$)
k	constant in Eq. (9)
k_c	constant in Eq. (1)
k_i	constant in Eq. (10)
$k_L a_L$	overall gas–liquid volumetric mass transfer coefficient (s^{-1})
K	consistency index ($N\,m^{-2}\,s^n$)
M	torque (N m)
n	flow behavior index
N	rotational speed of impeller (s^{-1})
N_p	Power number
N_{pg}	gassed Power number
P	unaerated power input (W)
P_g	gassed power input (W)
Re_g	generalized Reynolds number
t_c	circulation time (s)
T	tank diameter (m)
u_t	impeller tip speed ($m\,s^{-1}$)
V_L	volume of broth (m^3)
W	impeller blade height (m)

Greek letters

β	slope of linear plot of M versus N
ε	energy input per unit mass ($W\,kg^{-1}$)
γ_{av}	average shear rate (s^{-1})
μ	viscosity of Newtonian fluid ($N\,m^{-2}\,s$)
μ_a	apparent viscosity of non-Newtonian fluid ($N\,m^{-2}\,s$)
ρ	density of fluid ($kg\,m^{-3}$)
τ_{av}	average shear stress ($N\,m^{-2}$)

Mevinolin, Monacolin K and Mevacor™). In submerged cultures, fungi can be grown as broths of freely suspended mycelia and pellets or clumps (Metz and Kossen, 1977). For a given total biomass concentration, pelleted growth produces broths that are relatively less viscous and therefore easy to mix and aerate (Metz et al., 1979). The specific growth morphology obtained under given conditions depends on several factors including the fungal strain, the method of initiation of culture (e.g. spores, pellets, dispersed mycelium), the nature of the growth medium, and the hydrodynamic regime in the bioreactor (Metz and Kossen, 1977; Metz et al., 1979; van Suijdam and Metz, 1981). Excessive hydrodynamic shear stresses are known to damage mycelial hyphae and pellets (Chisti, 1999), but much lower shear stresses are sufficient to influence growth morphology (Metz and Kossen, 1977; van Suijdam and Metz, 1981; Chisti, 1999).

This work is concerned with the relationships among fermentation conditions, fungal morphology and the production of lovastatin by the microfungus *Aspergillus terreus*. Fermentation-derived lovastatin is also a precursor for simvastatin (Daborah et al., 1992), a powerful semi-synthetic statin commercially available as Zocor™. *A. terreus* produces lovastatin as a secondary metabolite and is commercially used to produce this drug (Novak et al., 1997; Szakács et al., 1998; Manzoni et al., 1998, 1999). *A. terreus* batch cultures are typically carried out at ~28 °C and pH 5.8–6.3 (Kumar et al., 2000). The dissolved oxygen level is controlled at ≥40% of air saturation (Kumar et al., 2000). A batch culture generally runs for <10 days.

In submerged cultures, *A. terreus* can grow in a variety of morphological forms, varying between a network of freely dispersed mycelia to tightly packed, discrete pellets. The culture conditions and the inoculation method seem to determine the morphological form. At least in some cases, pelleted growth has yielded higher titers of lovastatin than obtained with dispersed filamentous growth (Kumar et al., 2000). Dispersed filamentous morphology develops when the fungus grows on rapidly metabolized substrates. The rapid increase in viscosity that accompanies filamentous growth greatly impedes oxygen transfer and this is said to explain the low titers of lovastatin produced under dispersed growth conditions. Although many publications have reported on the effects of mechanical forces on fungal morphology and productivity (Moo-Young and Chisti, 1988; Moo-Young et al., 1992, 1993; Jüsten et al., 1996, 1998; Amanullah et al., 1999; Li et al., 2000, 2002), there is no specific data on *A. terreus* fermentations. In general, little is known about the influence of shear stresses on pelleted fungal cultures (Cui et al., 1997).

In the present work, *A. terreus* ATCC 20542 was used to produce lovastatin by batch culture. Our previous work with this fungus identified the optimal culture medium for producing lovastatin (Casas López et al., 2003, 2004). The effects of availability of oxygen and other nutrients (C, N, P) on the titer of lovastatin were established. Oxygen was found to be the major factor that influenced the lovastatin concentration. Lovastatin titer increased by approximately fourfold when the oxygen content of the aeration gas was changed from 20% (v/v) to 80% (Casas López et al., 2004). The present study aimed to decipher the relationships among lovastatin production, pellet morphology and the broth rheology as affected by mechanical agitation in the bioreactor. To separate the influence of agitation from the oxygen mass transfer effects, batch fermentations were carried out at different agitation intensities while the dissolved oxygen concentration was maintained at 400% of air saturation. In addition, to verify the enhancement of lovastatin titers by oxygen, control runs were carried out at 300 and 800 rpm agitation speeds while sparging with standard air. Pellet morphology, bulk broth rheology and lovastatin titers were measured under various aeration–agitation regimens.

2. Materials and methods

2.1. Microorganism and inoculation

A. terreus ATCC 20542 was obtained from the American Type Culture Collection. The fungus was maintained in Petri dishes of PDA (potato dextrose agar). 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 ($\sim 2800 \times g$, 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 that had been made with a flow cytometer (Coulter Epics XL-MCL).

2.2. Growth conditions

Fungal pellets were obtained by germination from spores suspended in shake flasks in a preliminary cultivation stage, and used for further inoculation of a stirred tank bioreactor. 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. For all experiments, a seed culture flask (250 mL) was used to inoculate a 5-L working volume bioreactor operated at 28 °C. Cultivations lasted around 7 days. The culture medium contained lactose as carbon source and soybean meal as nitrogen source. The medium contained per liter: 114.26 g of lactose, 5.41 g of soybean meal, 0.8 g of KH_2PO_4, 0.4 g of NaCl, 0.52 g of $MgSO_4{\cdot}7H_2O$, 1 mg of $ZnSO_4{\cdot}H_2O$, 2 mg of $Fe(NO_3)_3{\cdot}9H_2O$, 0.04 mg of biotin and 1 mL of a trace element solution. The trace element solution contained (for 1 L of solution): $Na_2B_4O_7{\cdot}10H_2O$, 100 mg; $MnCl_2{\cdot}4H_2O$, 50 mg; $Na_2MoO_4{\cdot}2H_2O$, 50 mg and $CuSO_4{\cdot}5H_2O$, 250 mg. The initial pH was adjusted to 6 with 0.1 M NaOH. In the oxygen-enriched experiments, dissolved oxygen concentration in the liquid was controlled at 400% of air saturation by maintaining an oxygen content of about 80% in the gas phase by supplementing it with pure O_2. Gas phase was recirculated in a closed loop and the generated CO_2 was absorbed in a $Ba(OH)_2$ solution in order to keep CO_2 level below 0.1% (v/v). Oxygen and CO_2 partial pressures in the gas stream were measured with an on-line sensor (Adaptive Biosystems Ltd., UK).

2.3. Stirred tank fermentations

Fermentations were conducted in a 5-L working volume bioreactor (Bioflo III, New Brunswick Co., USA) with a vessel internal diameter, T, of 0.17 m, four baffles, rounded bottom and a broth height to vessel diameter ratio of 1.4. Agitation was provided by two Rushton turbines with D/T ratio of 0.38 and W/D ratio of 0.18. Spacing between the impellers was $2D$ and the lower impeller was located at a distance D above the base of the tank. Agitation speed values of 300, 600 and 800 rpm were used. A pipe sparger aerated the culture at 1 vvm. At this value of aeration rate, gassed and ungassed agitation power inputs were measured off-line for various pelleted culture broths using a IKA®-Werke EUROSTAR power control device

(IKA® Werke GmbH & Co., KG, Staufen, Germany). Where necessary, the impeller power was expressed as the energy dissipation/circulation function (EDCF), defined as follows:

$$\mathrm{EDCF} = \frac{P_g}{k_c D^3 t_c} \tag{1}$$

where P_g is the gassed power input. In Eq. (1), the geometric constant k_c was determined as reported by Jüsten et al. (1996) and the gassed circulation time t_c was calculated following Jüsten et al. (1998).

2.4. Rheological measurements

Rheological parameters (K, n) were measured using a programmable rotational viscometer (Brookfield DV-II+ with standard vane spindle V-72, 21.67 mm diameter × 43.33 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.

The theory of rheological measurements with the "cup and vane" rotational viscometer as used here, is well known (Roels et al., 1974; Metz et al., 1979; Kemblowski and Kristiansen, 1986). A rheogram of the culture broth of interest is obtained as a plot of the average shear stress τ_{av} measured at different average shear rate (γ_{av}) values. The calculations of the flow index (n) and the consistency index (K) involved a novel approach, as presented next.

The impeller Power number (N_p) and Reynolds number (Re_g) for a mixing device operating in laminar flow are related as follows (Rushton et al., 1950a,b):

$$N_p = \frac{c}{Re_g} \tag{2}$$

where the constant c depends on the specific geometry of the mixing system. In Eq. (2), N_p and Re_g are the Power number and the generalized Reynolds number defined as follows:

$$N_p = \frac{P}{\rho N^3 D^5} \tag{3}$$

$$Re_g = \frac{\rho N D^2}{\mu_a} \tag{4}$$

In these equations, P is the agitation power, ρ the density of the fluid, μ_a the apparent viscosity, N the rotational speed of the agitator and D is its diameter.

Substitution of the definitions of the Power number (Eq. (3)) and Reynolds number (Eq. (4)) in Eq. (2) leads to the following equation:

$$\frac{P}{\rho N^3 D^5} = \frac{c\mu_a}{\rho N D^2} \tag{5}$$

The power input P is related to the measured torque (M) on the impeller and the rotational speed, as follows:

$$P = 2\pi NM \tag{6}$$

Eqs. (5) and (6) lead to the following expression for the apparent viscosity:

$$\mu_a = \frac{2\pi M}{cND^3} \tag{7}$$

However, by definition, the apparent viscosity is the average shear stress (τ_{av}) divided by the average shear rate (γ_{av}), or

$$\mu_a = \frac{\tau_{av}}{\gamma_{av}} \tag{8}$$

Furthermore, the average shear rate for Newtonian liquids agitated in a stirred tank under laminar flow is given as follows (Metzner and Otto, 1957):

$$\gamma_{av} = kN \tag{9}$$

where the constant k depends on impeller type and geometry. Similarly, for non-Newtonian liquids, the average shear rate depends on the agitation speed, as follows (Calderbank and Moo-Young, 1959):

$$\gamma_{av} = k_i \left(\frac{4n}{3n+1}\right)^{n/(n-1)} N \tag{10}$$

where k_i is a constant.

Eqs. (7)–(10) can be used to obtain the following relationships for the average shear stress:

$$\tau_{av} = \frac{2\pi kM}{cD^3} \tag{11}$$

for Newtonian fluids and

$$\tau_{av} = \frac{2\pi k_i \left(\frac{4n}{3n+1}\right)^{n/(n-1)} M}{cD^3} \tag{12}$$

for non-Newtonian media. Use of Eqs. (11) and (12) requires the values of the viscometer constants c, k, and k_i.

2.4.1. Determination of constants c and k_i

From Eqs. (8), (9) and (11) for Newtonian flow, we have

$$M = \frac{\mu c D^3}{2\pi} N \qquad (13)$$

Thus, for a given impeller viscometer and a Newtonian liquid of known viscosity, the torque M measured at several values of the rotational speed N, can be used to determine the constant c from the slope of a linear plot of M versus N. If the slope of such a plot is β, then c is given by

$$c = \frac{2\pi\beta}{\mu D^3} \qquad (14)$$

The Newtonian liquid used for estimating c was silicon oil ($\mu = 0.100\ \mathrm{N\,m^{-2}\,s}$ at 25 °C).

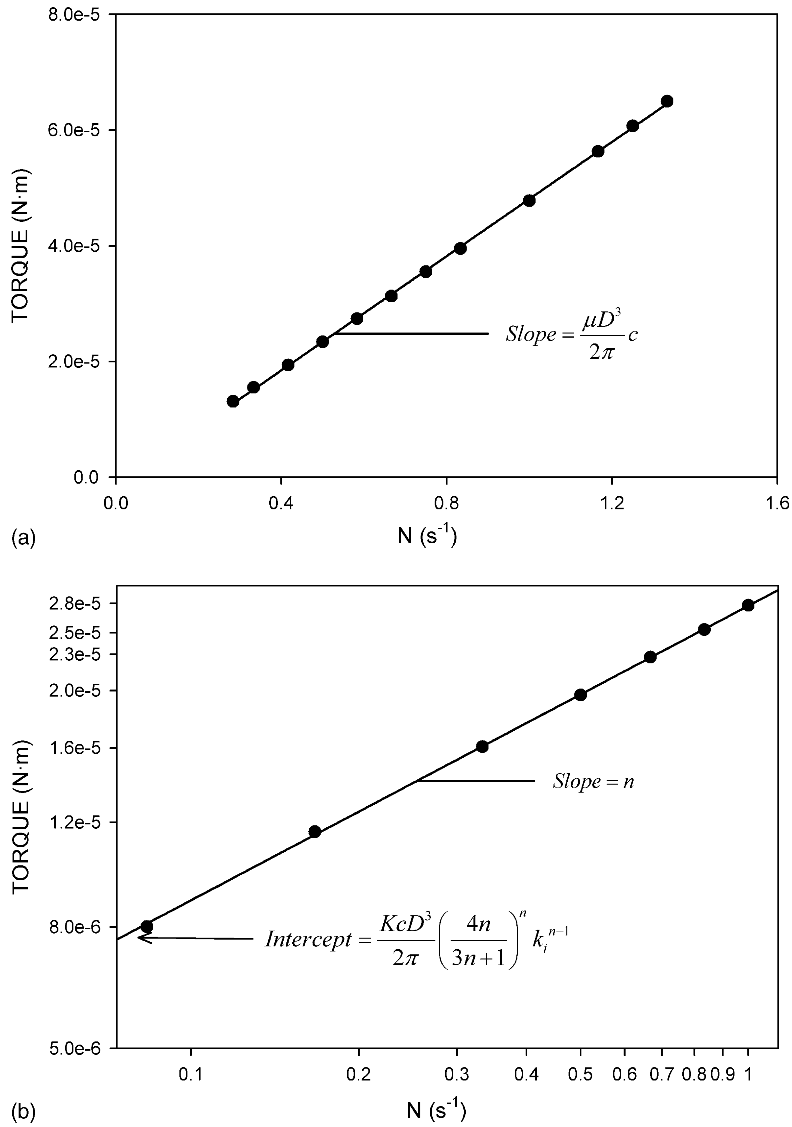

Fig. 1. Torque vs. rotational speed of viscometer: (a) linear plot for silicon oil (Newtonian) and (b) log–log plot for the non-Newtonian xanthan gum (0.1%).

The constant k_i (Eq. (10)) is determined using a non-Newtonian liquid of known rheological properties obeying the Ostwald–deWaele power law model, i.e.

$$\tau_{av} = K\gamma_{av}^n \tag{15}$$

In this case, the apparent viscosity is

$$\mu_a = \frac{\tau_{av}}{\gamma_{av}} = K(\gamma_{av})^{n-1} \tag{16}$$

By substituting in Eq. (16) the relevant expressions of μ_a (Eq. (7)) and γ_{av} (Eq. (12)) for non-Newtonian fluids, the following is obtained:

$$M = AN^n \tag{17}$$

where

$$A = \frac{KcD^3}{2\pi}\left(\frac{4n}{3n+1}\right)^n k_i^{n-1} \tag{18}$$

Thus, a log–log plot of M versus N should be a straight line with the slope n and the y-intercept A. From the intercept A and the known consistency index K, the value of constant k_i can be calculated. The known non-Newtonian liquid used for estimating the k_i value was a 0.1% (w/v) aqueous solution of xanthan gum ($K = 0.178\ \mathrm{N\,m^{-2}\,s^n}$; $n = 0.495$).

The plots of Eqs. (14) and (17), are shown in Fig. 1 for Newtonian and non-Newtonian fluids, respectively. The estimated c and k_i values for the viscometer were 301.84 and 7.599, respectively. The regression coefficients (r^2) values for the plots in Fig. 1 were better than 0.999.

2.4.2. Determination of K and n of fermentation broth

For any non-Newtonian fluid of unknown rheological variables K and n, measurements of torque (M) for several values of the rotational speed N with a calibrated viscometer (i.e. known c and k_i) allow the construction of a log–log plot of M versus N (Fig. 2). The slope of this plot is the desired n-value and its y-intercept provides log A that is related to the consistency index K (Eq. (18)). These measurements must be made in the laminar flow regime. In a stirred tank, the flow is generally accepted to be laminar when the value of the generalized Reynolds number Re_g is less than 10; however, Re_g values approaching 60 have been associated with laminar flow (Kemblowski and Kristiansen, 1986). In view of the uncertainties, a preferred approach is to always demonstrate the existence of laminar flow over the measurement range with the specific fluid being measured. This can be done easily: for laminar flow, a log–log plot of the Power number versus the generalized Reynolds number should have a slope of −1.

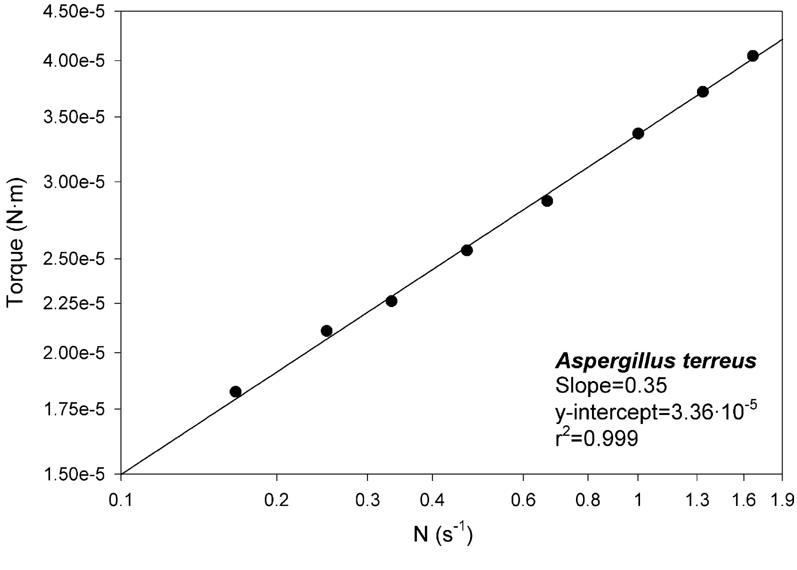

Fig. 2. Torque vs. rotational speed (spindle V-72) plot for a broth of *A. terreus* ($n = 0.35$, $K = 0.296\ \mathrm{N\,m^{-2}\,s^n}$).

2.5. Morphological measurements

The fungal pellet morphology was characterized using image analysis (Paul and Thomas, 1998). Prior to imaging, each sample of the 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) that used a 40× magnification. Image analysis was performed with the software package Image-Pro Plus 4.5.1 (Media Cybernetics Inc., Silver Spring, MD, USA).

Previous studies have characterized fungal pellets as consisting of a central compact core region and a peripheral filamentous or hairy region (Cui et al., 1997). Thus, the morphology has been characterized in terms of a pellet core diameter (i.e. the equivalent diameter of the measured core area) and the width of the hairy zone. The latter is obtained by subtracting the equivalent radius of the core from the total equivalent radius of the pellet (Cui et al., 1997).

A similar approach was used in the present study. The 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 projected 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 cultivation, a young pellet would be typically characterized by a small diameter and a filament ratio close to 100%. As the experiment progressed, the filament ratio would reduce.

2.6. Analytical methods

2.6.1. 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.

2.6.2. Lovastatin

Lovastatin was measured in its β-hydroxyacid form by HPLC of the biomass-free filtered broth (Friedrich et al., 1995; Morovján et al., 1997). 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 ., SA) 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 0.1N 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 mm × 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. 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.

3. Results and discussion

The measured mechanical power inputs in the gassed and ungassed states were used to construct the culture system power curves shown in Fig. 3. The curves matched the trends expected from the literature (Jüsten et al., 1998). For gassed Power number (N_{pg}) at the lowest impeller rotational speeds, torque measures were unstable and a precise determination of power input was not possible.

3.1. Pellet morphology and broth rheology

After inoculation of the bioreactor with a seed culture of pellets of ∼1200 μm mean diameter, the growth and fragmentation patterns observed are shown in Fig. 4. Irrespective of whether air or oxygen-enriched gas was used for aeration, the pellets grew in size only when the agitation speed was 300 rpm (impeller tip speed, u_t = 1.02 m s^{-1}). Higher agitation speeds caused fragmentation of pellets. At 300 rpm agitation speed, the pellet size at steady state almost doubled to a value of about 2300 μm. At agitation intensities of 600 (u_t = 2.03 m s^{-1}) and 800 rpm (u_t = 2.71 m s^{-1}), the

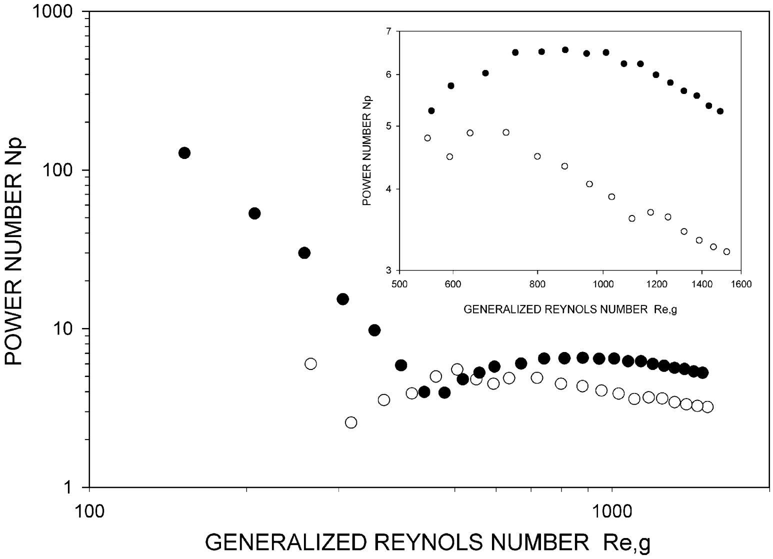

Fig. 3. Power curves for ungassed (filled symbols) and aerated (open symbols) culture broth.

pellet size declined from the instance of inoculation to attain an average steady state diameter of around 700 μm.

The steady state size of the microbial aggregates and fungal pellets suspended in mechanically agitated tanks is known to decline with the increasing energy input in the tank and the tip speed of the impeller (Cui et al., 1998; Chisti, 1999). The known mechanisms of size reduction include surface erosion of the pellet and its outright rupture (Cui et al., 1997). How rapidly the pellet size declines to the steady state value depends also on the initial size of the pellet (Chisti, 1999). As the decline in pellet size (Fig. 4) was gradual and progressive, it was likely caused by erosion

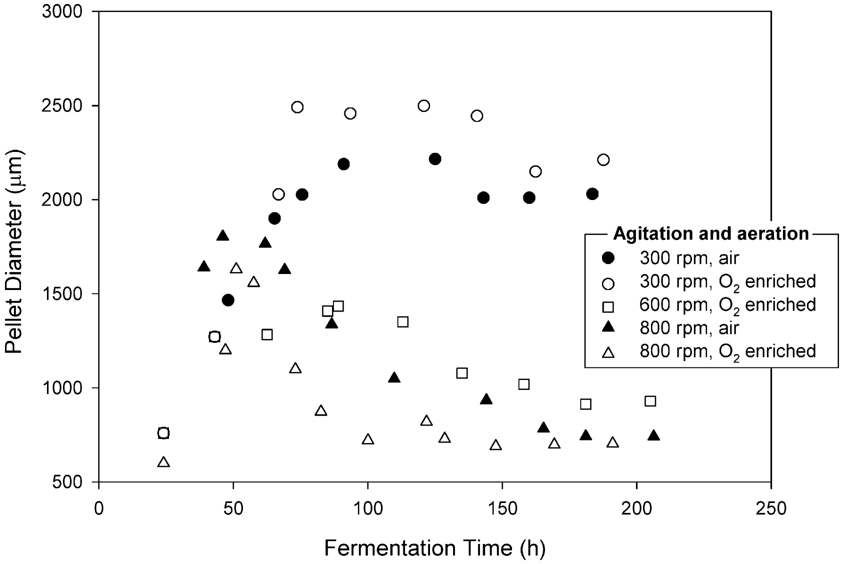

Fig. 4. Pellet diameter obtained at various times, agitation speeds and gas-phase oxygen concentrations.

of the pellet surface rather than outright rupture of the pellet. At the low agitation intensity of 300 rpm, the pellets grew in size because the Kolmogoroff microscale of turbulence was larger than the pellet diameter of 1200 μm and the erosion of the surface did not come into effect until the pellets had attained a size of approximately 2500 μm. Following similar trends, under extremely low intensity agitation such as in non-mechanically agitated airlift bioreactors, fungal pellets can attain a size of several centimeters, but such pellets are internally hollow because of a necrosis of the fugal biomass caused by a lack of oxygen inside the pellet.

The measured filament ratio is shown in Fig. 5 for constant intensities of agitation during various cultivations. At inoculation, the 1200 μm diameter pellets had mostly a loose hairy morphology with a filament ratio of near unity. The filament ratio declined with time at all agitation intensities (Fig. 5) even though the agitation rate of 300 rpm did not damage the pellets (Fig. 4). Thus, even low-level agitation tended to favor a reduction in the filament ratio apparently because the fluid eddies were forcing and folding the peripheral hyphae into the pellet and physically compacting the structure of the pellet. Fig. 5 suggests no clear influence of a high oxygen environment on the development of the compact morphology and, therefore, the observed reduction in the filament ratio is likely purely an effect of the fluid mechanical forces.

Both the biomass concentration and morphology are of course well known to influence the rheological properties of mycelial fermentations broths (Metz and Kossen, 1977; Metz et al., 1979). Furthermore, the dispersed filamentous growth is well documented to produce broths that a more viscous and rheologically different compared to pelleted growth with an equal biomass concentration. How the morphology of the pellet influences rheology is not so well known.

Fig. 6a–c shows the variations in the consistency index, the flow behavior index and the apparent viscosity, respectively, of the broth during the course of the experiments carried out at low (i.e. 300 rpm) and high (i.e. 800 rpm) agitation intensities while using two different modes of oxygen supply. The figures reveal some important features of the broth rheology: (1) At the low agitation rate of 300 rpm (average shear rate $\approx 70\ s^{-1}$), the K-value (i.e. the "thickness" of the fluid) increased substantially as the biomass concentration (Fig. 7) and the pellet size increased (Fig. 4). (2) At a high-intensity agitation of 800 rpm (average shear rate $\approx 198\ s^{-1}$), the pellet size declined with time (Fig. 4), the biomass concentration increased (Fig. 7), but the K-value remained quite steady at approximately $0.01\ N\,m^{-2}\,s^{n}$ throughout the fermentation (Fig. 6a). This suggests that the K-

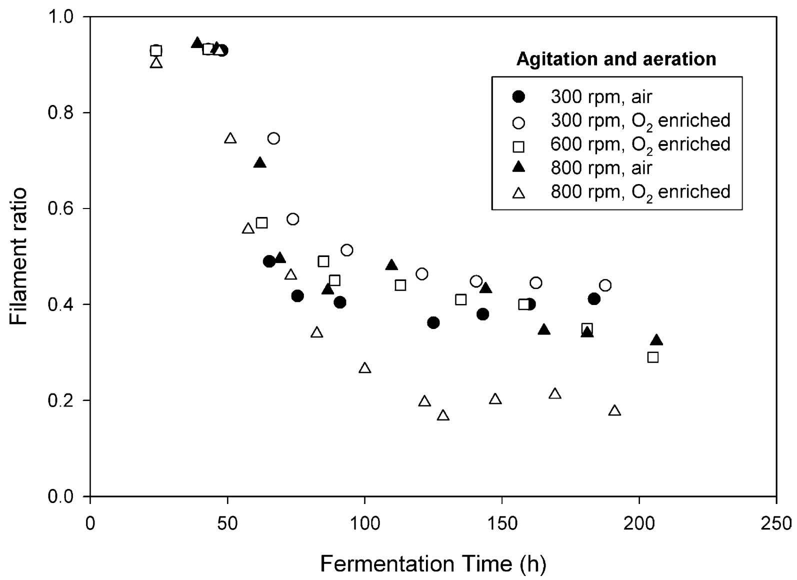

Fig. 5. Filament ratio vs. fermentation time at various agitation speeds and oxygen concentrations.

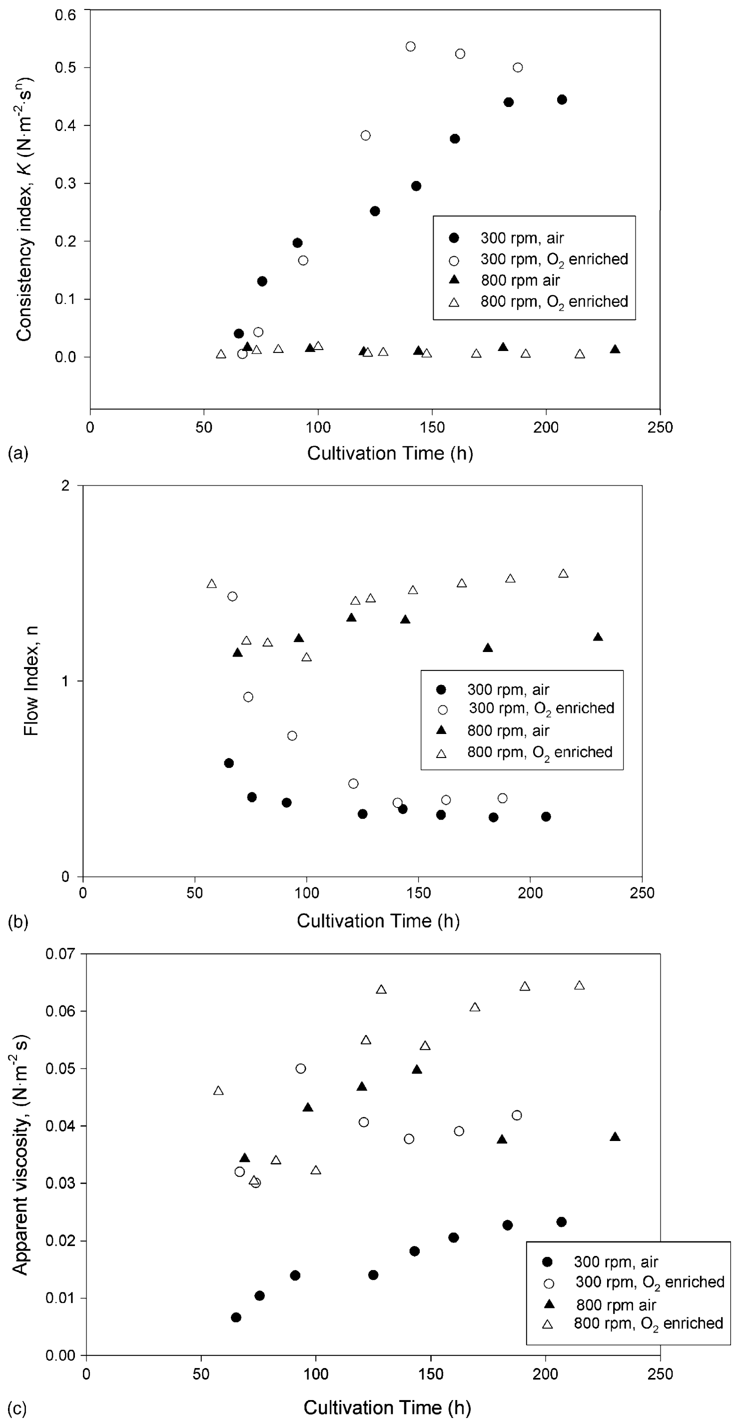

Fig. 6. Broth rheology vs. fermentation time: (a) consistency index, K; (b) flow index, n; (c) apparent viscosity.

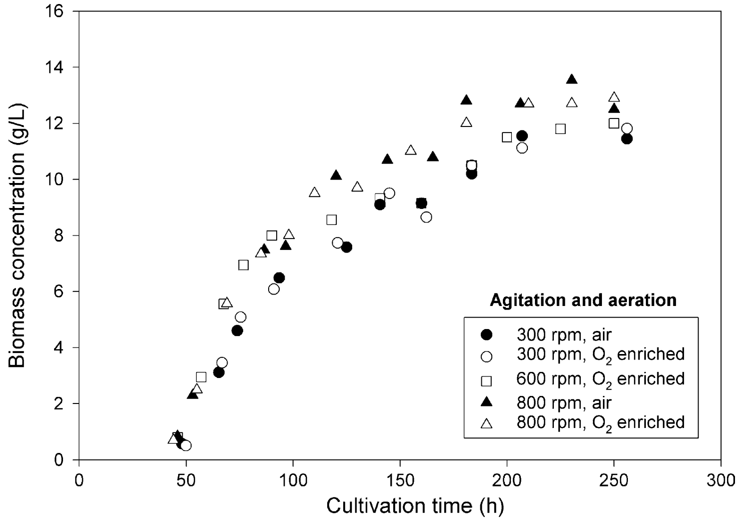

Fig. 7. Biomass growth curves for different agitation and aeration regimens.

value of broths containing small pellets (e.g. <900 μm in diameter) is not particularly sensitive to an increase in the concentration of the pellets. (3) The flow index n declined strongly as the biomass concentration increased with time (Fig. 6b) in the low shear fermentation (300 rpm). In contrast, the n-value did not vary much during the experiments conducted at the high agitation speed. This suggests that a reducing pellet diameter below 1200 μm (Fig. 4) and an increasing biomass concentration (Fig. 7) mutually counteracted so that the n-value did not vary much with time. The small pellets formed at the high agitation speed produced broths with n-values exceeding unity, i.e. shear thickening broths. This is in complete contrast to the shear thinning or pseudoplastic broths that are generally observed for dispersed mycelial growth (Chisti, 1989). Broths of the large fluffy pellets obtained at low-intensity agitation generally had a shear thinning behavior (i.e. $n < 1$) (Fig. 6b). How the broths were aerated (i.e. with oxygen-enriched gas or air) had little impact on the K- and n-values (Fig. 6) of the broth or the biomass growth patterns (Fig. 7), suggesting that aeration was always sufficient for biomass growth.

To facilitate the comparison among the broths produced under different conditions, the apparent viscosity calculated at the estimated average shear rate (Eq. (10)) for each impeller rotational speed, is shown in Fig. 6c for the various broths. Generally, because of the shear thickening behavior, the broths produced at the higher agitation rate were more viscous than the ones obtained at the lower agitation speed.

The differences in the bulk broth rheology seen in Fig. 6 could not be ascribed to possible differences in the total biomass concentrations of the broths. This was because there was no statistical difference in the biomass concentration profiles for the five combinations of the agitation–aeration conditions assessed in the fermentations (Fig. 7). All experiments commenced with the same concentration of the inoculum biomass and morphology. Irrespective of the agitation intensity and the mode of oxygen supply used, all fermentations attained a final average dry biomass concentration of $11.9 \pm 0.4\,\mathrm{g\,L^{-1}}$ (95% confidence level) (Fig. 7).

A multifactor analysis of variance was carried out to establish the extent of influence of the biomass concentration and the morphological parameters of the pellet, on the broth rheology (i.e. K and n). This is shown in Table 1. As the P-values for pellet diameter and filament ratio were less than 0.05 (Table 1), these factors had statistically significant effects on the consistency index and flow behavior index, at the 95% level of certainty. The pellet diameter was the main factor that influenced K and n. It seems, therefore, that the biomass concentration affects K and n mainly when the growth morphology is of the dispersed type, as shown in many previous studies (Metz et al., 1979; Chisti, 1989; Riley

Table 1
Analysis of variance for power law parameters

Source	Consistency index, *K*					Flow behavior index, *n*				
	Sum of squares	d.f.	Mean square	*F*-ratio	*P*-value	Sum of squares	d.f.	Mean square	*F*-ratio	*P*-value
Biomass concentration	0.0515	2	0.0257	2.14	0.1419	0.0003	2	0.0002	0.00	0.9980
Mean pellet diameter	0.2425	2	0.1212	10.07	0.0008	1.2284	2	0.6142	8.09	0.0023
Mean filament ratio	0.1117	2	0.0558	4.64	0.0209	0.9901	2	0.4951	6.52	0.0060
Residual	0.2649	22	0.0120			1.6709	22	0.0759		
Total (corrected)	0.8407	28				5.8370	28			

All *F*-ratios are based on the residual mean square error.

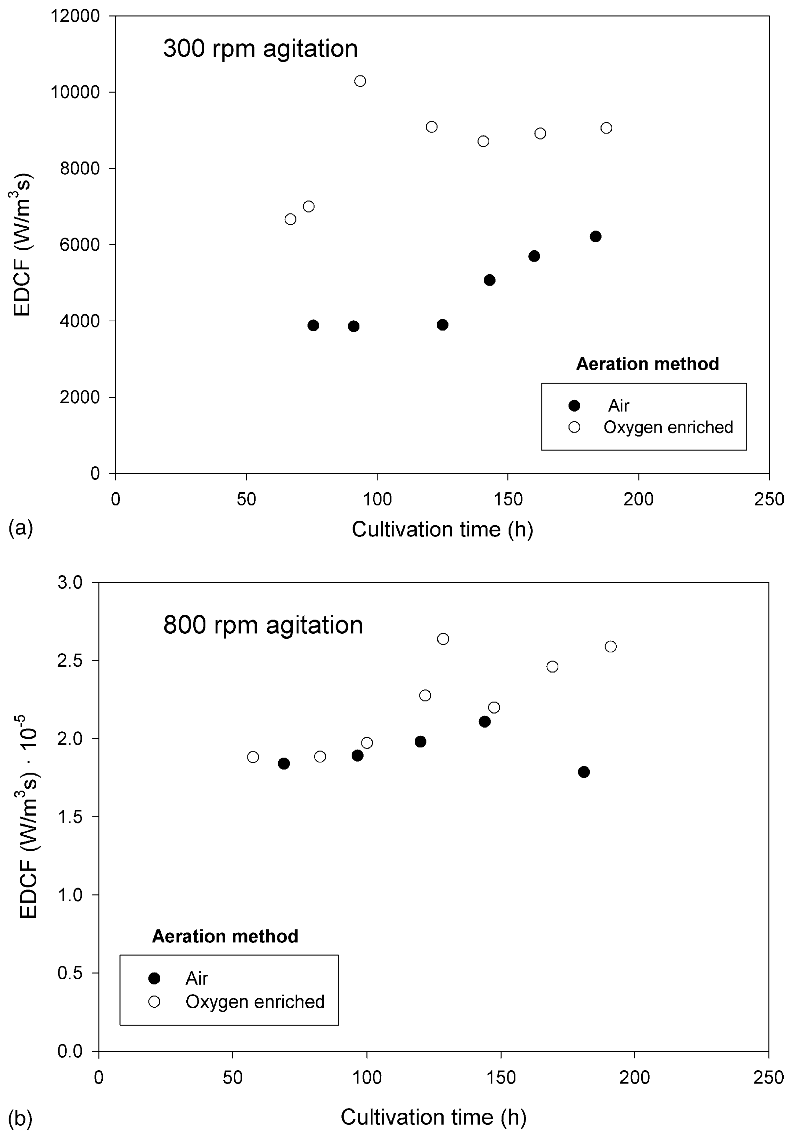

Fig. 8. Energy dissipation/circulation function (EDCF) vs. fermentation time for cultures agitated at: (a) 300 rpm and (b) 800 rpm.

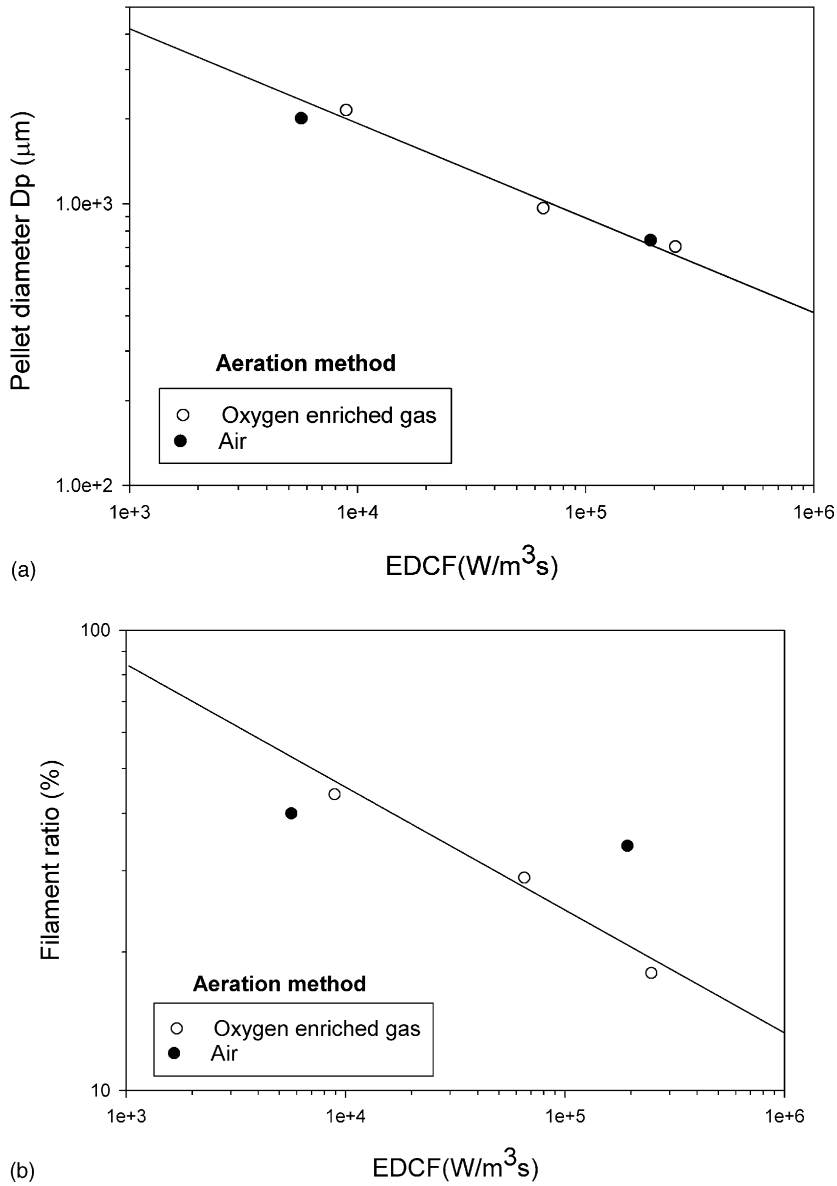

Fig. 9. Correlation of the morphological variables D_P (a) and filament ratio (b) with EDCF. The morphology was quantified at the end of the fermentations.

et al., 2000; Papagianni, 2004), but biomass concentration has relatively little effect on rheology in pelleted growth.

In the past, mycelial morphology and damage to other suspended particles have been correlated with the energy dissipation/circulation function (EDCF) (Smith et al., 1990; Makagiansar et al., 1993; Chisti, 1999). EDCF recognizes that disruption of particles suspended in a stirred vessel is a function of the energy dissipation rate in the impeller zone and the frequency at which the particles circulate through this zone. Morphological data have been correlated with EDCF for tanks operating at different scales and with impellers of very different geometries (Jüsten et al., 1996, 1998;

Table 2
Empirical correlations for the mean pellet diameter, D_P

Correlator	Model	Determination coefficient, r^2
EDCF ($kW\,m^{-3}\,s^{-1}$)	D_P (μm) = 4383 (EDCF)$^{-0.34}$	0.983
P_g/V_L ($kW\,m^{-3}$)	D_P (μm) = 929 $(P_g/V_L)^{-0.41}$	0.995
ε ($W\,kg^{-1}$)	D_P (μm) = 923 $\varepsilon^{-0.41}$	0.995

Amanullah et al., 1999; Li et al., 2000, 2002). In a study with pellets, Cui et al. (1997) used specific energy dissipation rate ε for correlating the pellet morphology.

The EDCF values calculated (Eq. (1)) at different fermentation times for operations at 300 and 800 rpm, are shown in Fig. 8. For any given fermentation, the EDCF value varied with time (because of changes in K and n), but not substantially. Considering this, an average EDCF value can be taken as representative of a given fermentation. If the average pellet diameter and the filament ratio measured at the end of a fermentation are correlated with the average EDCF, we obtain Fig. 9. The log–log correlations in Fig. 9 for data from five different fermentations, suggest that EDCF might indeed be a good variable for describing the influence of stirred tank hydrodynamics on pellet morphology. Other work has successfully correlated the mean projected area of pellets, with EDCF (Jüsten et al., 1996, 1998). However, as shown in Table 2, parameters such as D_P can be correlated quite satisfactorily with the specific energy input (i.e. P_g/V_L or ε) in the tank. The observed exponent of −0.41 on the specific energy input term (Table 2) was close to the value of −0.36 reported by van Suijdam and Metz (1981) and to the theoretical value of −2/5 (Cui et al., 1997). A very different value of −0.16 was reported for this exponent by Cui et al. (1997) in cultures of *Aspergillus awamori*. This suggests that the mechanical strength of a pellet might depend on the fungal strain and possibly the growth conditions used in culturing it. Indeed, the strength of cell walls is known to be affected by both the microbial strain and the growth conditions (Chisti and Moo-Young, 1986).

3.2. Oxygen transfer

The oxygen transfer rate was evaluated as the overall volumetric oxygen transfer coefficient, k_La_L, obtained by the application of the dynamic method as reported by Taguchi and Humphrey (1966). As expected, k_La_L increased with agitation speed but varied little during a fermentation (Fig. 10). Consequently, an average k_La_L value was calculated as being representative of a given fermentation. These k_La_L values could be correlated with the EDCF and P_g/V_L values (Table 3). No fermentation was limited by oxygen supply, as they all had virtually identical biomass growth

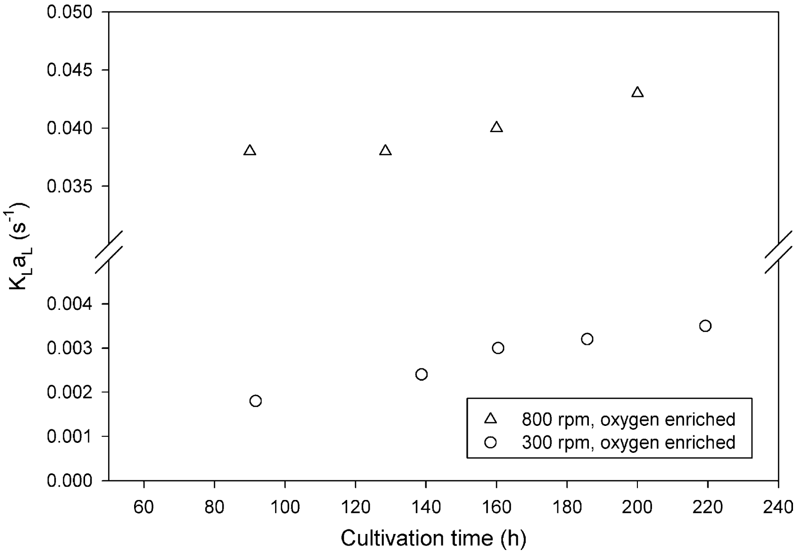

Fig. 10. Variation of the overall volumetric oxygen transfer coefficient, k_La_L, during the fermentation.

Table 3
Empirical correlations for the volumetric oxygen transfer coefficient, $k_L a_L$

Correlator	Model	Determination coefficient, r^2
EDCF (kW m^{-3} s^{-1})	$k_L a_L$ (s^{-1}) = 7.0 × 10^{-4} (EDCF)$^{0.76}$	0.975
P_g/V_L (kW m^{-3})	$k_L a_L$ (s^{-1}) = 2.24 × 10^{-2} $(P_g/V_L)^{0.92}$	0.991

curves (Fig. 7). However, the oxygen supply did affect the production of lovastatin, as discussed in the next section.

3.3. Lovastatin production

Shear rate, i.e. the agitation speed, affected the lovastatin titers produced (Fig. 11) even though the biomass concentration was not affected (Fig. 7). Thus, high lovastatin titers were attained in oxygen-enriched fermentations at 300 and 600 rpm. Comparable titers at 300 and 600 rpm suggest that oxygen transfer did not limit lovastatin synthesis at agitation speeds of ≥300 rpm (Fig. 11). However, in oxygen-enriched cultures, agitation at 800 rpm reduced lovastatin titers to the level of air-sparged cultures (Fig. 11). Clearly, while a high concentration of oxygen was essential to attain a high titer of lovastatin ($C_{24}H_{36}O_5$), a relatively oxygen-rich molecule, excessive shear did inhibit lovastatin synthesis but not the production of the biomass. Cultures aerated with air did not attain high titers at any agitation rate (Fig. 11) even though their biomass production was not limited by oxygen (Fig. 7). The negative impact of high shear on lovastatin titers is clearly seen in Fig. 12 where the final titer versus EDCF data for the various fermentations are plotted.

These results affirmed the earlier observations of increased lovastatin titers in oxygen-enriched fermentations compared to the fermentations sparged with air (Casas López et al., 2004); however, the observed decline in titers at the high agitation intensity was unexpected. Furthermore, at 80 mg L^{-1}, the maximum concentration of lovastatin attained in Fig. 11 was only about 30% of the 230 mg L^{-1} that was obtained in shake flasks (Casas López et al., 2004). As the biomass production was not affected by agitation speed in the 300–800 rpm range (Fig. 7), a possible interactive effect of oxygen tension and growth morphology appears to influence the production of lovastatin. At 800 rpm, the beneficial effect of higher oxygen supply is lost because of the shear-induced alterations to pellet morphology.

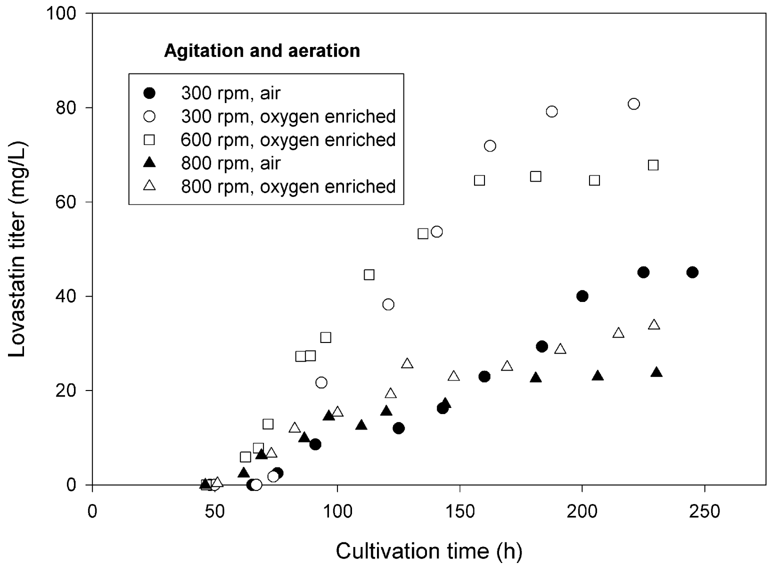

Fig. 11. Lovastatin concentration vs. fermentation time for various agitation speeds and aeration regimens.

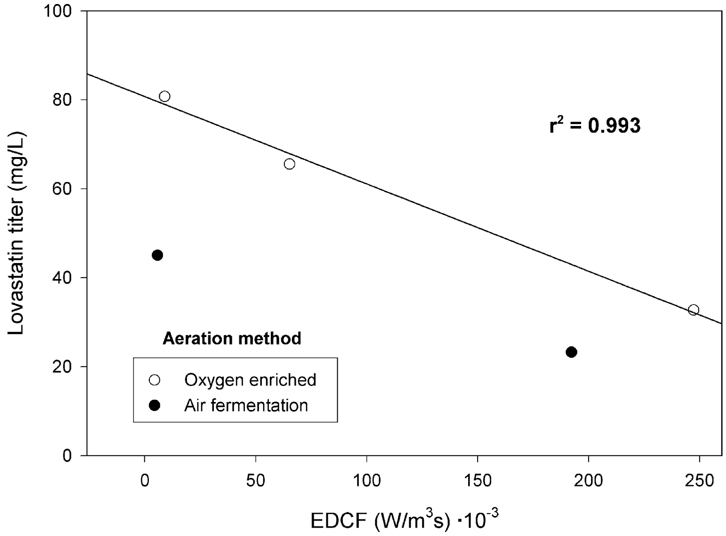

Fig. 12. Final lovastatin concentration vs. EDCF in variously aerated fermentations.

At 800 rpm in oxygen-enriched cultures, the $k_L a_L$ was 15 times greater and average pellet size was three times smaller, than the values at 300 rpm, suggesting an enhanced oxygen transfer from the liquid bulk to the pellet. Nevertheless, the lovastatin titer decreased in the same proportion as the decrease in the filament ratio of the pellets. Lovastatin synthesis apparently occurred in the filamentous zone of the pellet and not in the central core. Large pellets with a high ratio of filament-to-core zones grown in an oxygen rich environment were the best for maximal production of lovastatin.

4. Conclusions

In pelleted growth of *A. terreus* in mechanically agitated submerged batch cultures, the biomass growth profiles were little affected by the agitation speed (300–800 rpm), or the mode of aeration (air or oxygen-enriched gas). However, the agitation speed significantly affected the pellet diameter, morphology and lovastatin production. Agitation speeds of $\geq$600 rpm damaged the fungal pellets of $\sim$1200 μm initial diameter, reducing them to a final stable pellet diameter of $\sim$900 μm. At lower agitation speeds, the stable maximum pellet diameter exceeded $\sim$2500 μm. Pellets of this size produced high lovastatin titers when aerated with oxygen-enriched gas but not with air. Much smaller pellets produced under intense agitation ($\geq$800 rpm) gave poor productivities of lovastatin, irrespective of the mode of aeration used. This suggests that a high oxygen concentration in the pellet is necessary but not sufficient for attaining a high titer of lovastatin. Pellets that had a relatively less dense, open, filamentous morphology were better at producing lovastatin compared to small denser pellets. Thus, both an upper limit on acceptable hydrodynamic shear stress and a high oxygen level are indicated for superior production of lovastatin.

Pellet size in the bioreactor correlated equally well with the specific energy input rate and the energy dissipation circulation function, EDCF. Broths of relatively small and dense pellets tended to have a shear thickening rheology. In contrast, broths of the relatively large fluffy pellets were strongly shear thinning.

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