World J Microbiol Biotechnol (2010) 26:1409–1418
DOI 10.1007/s11274-010-0315-9

ORIGINAL PAPER

Influence of ultrasound amplitude and duty cycle on fungal morphology and broth rheology of *Aspergillus terreus*

N. Sainz Herrán · J. L. Casas López · J. A. Sánchez Pérez · Y. Chisti

Received: 16 October 2009 / Accepted: 7 January 2010 / Published online: 19 January 2010
© Springer Science+Business Media B.V. 2010

Abstract Effects of ultrasound amplitude and duty cycle on cultures of *Aspergillus terreus* are reported in a 25 l slurry bubble column sonobioreactor. Fermentations were carried out batchwise. A 2^k-factorial design with added central points was used. Sonication at any cycle and amplitude level did not affect biomass growth rate and yield relative to nonsonicated control, but did affect growth morphology. Ultrasound disrupted fungal pellets and caused the biomass to grow mainly as dispersed hyphae. Production of lovastatin was reduced by medium- and high-cycle sonication. Sonication affected broth rheology. In view of these results, sonication can be used to modify growth morphology and broth rheology without affecting growth rate and yield of filamentous fungi.

Keywords *Aspergillus terreus* · Fungal rheology · Fungal morphology · Lovastatin · Sonobioreactors · Ultrasound

Introduction

Ultrasound, or sound of frequency >20 kHz, is commonly used to break microbial cells to release intracellular products (Chisti and Moo-Young 1986). Although high-intensity ultrasound breaks microbial cells, ultrasound of low intensity has been shown to improve productivity of at least some fermentation processes without damaging cells (Chisti 2003). Causes of this productivity enhancement are not entirely clear, but factors such as improved gas–liquid mass transfer and solid–liquid mass transfer appear to be involved. Ultrasonication may also influence metabolic processes in an otherwise undamaged cell. This work elaborates on some of the observed effects of ultrasound on *Aspergillus terreus* in batch fermentations for producing lovastatin (Sainz Herrán et al. 2008). The aim is to identify quantitatively how the final concentration of lovastatin is influenced by the amplitude and duty cycle (i.e. the duration of sonication within a period of one second) of sonication.

Magneto-restrictive or piezoelectric transducers are used to generate ultrasound. These transducers convert the alternating current of an electronic oscillator to mechanical waves that are transmitted to the microbial broth through a cylindrical rod-shaped probe, or sonotrode (Chisti 2003). Effects of sonication are associated with the energy it imparts to the culture broth (Chisti 2003). Most of the sonic energy is dissipated at the tip of the sonotrode. Low pressure is developed at the tip of the sonotrode during the rarefaction phase of the sound wave. This causes cavitation microbubbles to form and grow. During the subsequent compression phase, the bubbles implode, releasing a violent shock wave that propagates through the broth (Chisti 2003).

The filamentous fungus *A. terreus* is commercially used to produce lovastatin, a drug used in lowering the blood cholesterol. For many filamentous fungi, the growth morphology in a bioreactor is known to be influenced by the intensity of the prevailing hydrodynamic and mechanical

N. Sainz Herrán · J. L. Casas López (✉) · J. A. Sánchez Pérez
Department of Chemical Engineering, University of Almería, 04120 Almería, Spain
e-mail: jlcasas@ual.es

Y. Chisti
School of Engineering, Massey University, Private Bag 11 222, Palmerston North, New Zealand

shear forces (Casas López et al. 2005; Rodríguez Porcel et al. 2005; Sánchez Pérez et al. 2006). Growth morphology in turn affects metabolite productivity. For example, *A. terreus* produces higher titers of lovastatin when growing as pellets compared to when the growth morphology is predominantly of a dispersed filamentous type (Kumar et al. 2000; Casas López et al. 2005; Rodríguez Porcel et al. 2005; Sainz Herrán et al. 2008). For identical concentrations of biomass, a broth with mainly pelleted growth is typically much less viscous than a broth with predominantly dispersed filamentous growth. An elevated viscosity impedes oxygen transfer and this has been advanced as an explanation for the low titers of lovastatin during dispersed filamentous growth (Kumar et al. 2000). In view of its importance, lovastatin production by *A. terreus* has been extensively investigated (Novak et al. 1997; Szakács et al. 1998; Manzoni et al. 1998, 1999; Hajjaj et al. 2001; Lai et al. 2003; Casas López et al. 2003, 2004a, b, 2005; Rodríguez Porcel et al. 2005, 2006, 2007), but how ultrasound might influence this fermentation is barely known (Sainz Herrán et al. 2008). Some of the published information (Sainz Herrán et al. 2008; Casas López et al. 2003, 2004b, 2005) was used in selecting the suitable fermentation conditions for the present study. Ultrasonication for influencing microbial processes has been reviewed elsewhere (Chisti 2003).

Seven experiments were conducted for the study of the influence of different ultrasound intensities on *A. terreus* cultures. Two ultrasound operation variables (i.e. amplitude and cycle) were tested in several intensity value combinations to check their influence by a statistical design. Additionally a negative control culture was carried out.

Various methods of statistical experimental design have proved useful for studying the impact of many interacting variable on fermentation processes (Grothe et al. 1999). This work used a 2^k-factorial experimental design with added central points. A 2^k-design is a special case of factorial design that is useful in screening experiments for identifying the important system variables. The addition of central points in a 2^k-factorial design allows an independent estimate of error to be obtained.

The 2^k-factorial design with three central point replicates comprised two factors at three levels of variation to permit an unconfounded estimation of the regression coefficients. The factors and levels of variation were 20, 60 and 100% for the amplitude of ultrasound and 0.2, 0.6 and 1 s per second, for the cycle of application. (In percent terms, an ultrasound application cycle of 0.2 s per second was equivalent to 20%.) The responses were the yield of lovastatin, yield of biomass, growth rate of biomass (pellets and hyphal growth), and broth rheology (i.e. consistency index, *K*, and flow behavior index, *n*).

Materials and methods

Microorganism and preparation of inocula

Aspergillus terreus ATCC 20542 obtained from the American Type Culture Collection was used. The fungus was maintained on Petri dishes of PDA (potato dextrose agar). After inoculation, the dishes were incubated at 28°C for 5-days and then stored at 5°C. To obtain a suspension of spores, fungal growth from several Petri dishes was washed with a sterile aqueous solution of 2% Tween® 20. The resulting suspension was centrifuged (5,000×*g*, 3-min) and the spore pellet was resuspended in sterile distilled water. Optical density of the spore suspension was measured at 360 nm and converted to spore concentration using a standard curve. For preparing the standard curve, the optical density was correlated to the direct spore counts made using a flow cytometer (Coulter Epics XL-MCL; Beckman Coulter Ltd, UK).

All bioreactor fermentations were initiated by inoculation with a 850 ml suspension of fungal pellets. Pellet seed suspensions had been produced in 1,000 ml shake flasks by germination of a 300 ml spore suspension. Flasks inoculated with the spores were held on a rotary shaker at 150 rpm, 28°C, for 48-h. The culture medium contained per liter: 114.26 g of lactose (carbon source), 5.41 g of soybean meal (nitrogen source), 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.

Bioreactor fermentations

A slurry bubble column bioreactor (Fig. 1) of 25 l total volume (23 l of working volume) was used (Sainz Herrán et al. 2008). The reactor vessel had a diameter of 0.15 m and an aspect ratio of 6. Gas was sparged at 1 vvm using a perforated plate (150 holes of 1.5 mm diameter) that was located at the base of the reactor. The reactor vessel was jacketed for temperature control. The top degassing zone of the reactor column had a jacket of its own and this was kept at 4°C to prevent wall growth. Fermentations were carried out at 28°C. Each fermentation lasted 8-days. The power input due to isothermal expansion of the aeration gas was the same for each fermentation (213 W m^{-3}) and was estimated from the aeration rate by using the following equation (Chisti 1989):

$$\frac{P_g}{V_L} = \rho_L g U_g \quad (1)$$

where P_g (W) is the power input, V_L (m^3) is the volume of the broth in the bioreactor, ρ_L is the density of the broth (kg m^{-3}), g is the gravitational acceleration (m s^{-2}) and U_g (m s^{-1}) is the superficial aeration velocity. The dissolved oxygen concentration in the fermentation broth was maintained at 400% of air saturation by supplementing the sparged gas phase with pure oxygen. The gas leaving the reactor was passed through a solution of NaOH to absorb any carbon dioxide. This gas was then recirculated through the reactor in a closed loop. Pure oxygen was added to the recirculating gas to maintain the specified dissolved oxygen level in the reactor vessel. The CO_2 concentration in the recirculating gas remained below 0.1% v/v. Dissolved oxygen was measured online in the liquid phase using a polarographic electrode (Mettler Toledo; model InPro 6100/220T). Prior to inoculation, the dissolved oxygen electrode was calibrated in the sterilized culture medium. For this, the medium was sparged with air and once the dissolved oxygen reading had stabilized, the saturation value was set to 100%. In view of a fairly turbulent and steady state hydrodynamic regimen during operation, any effects of the liquid film at the surface of the electrode were disregarded, as is typical for this kind of work (Chisti 1999).

For operation as a sonobioreactor, a titanium sonotrode (22-mm in diameter, 300-mm long, model H22L3D; Dr. Hielscher GmbH, Stuttgart, Germany) was inserted in the above mentioned slurry bubble column at the headplate (Fig. 1) (Sainz Herrán et al. 2008). The sonotrode was connected to a generator with a fixed US frequency of 24 kHz (Dr. Hielscher GmbH, Stuttgart, Germany; model UP400S). The ultrasound power level was varied by adjusting the amplitude and the duration cycle of the sonotrode pulse between 20 and 100% (Sainz Herrán et al. 2008). A cycle time of 20%, for example, meant an ultrasound application period of 0.2 s every second. The sonotrode could deliver a maximum of 320 W of power with the amplitude and cycle time set to maximum values. The sonic power input was related directly to the square of the amplitude and to the cycle time. At the maximum power setting, 62.5% of the power was transmitted to the broth from the tip of the sonotrode and the rest was transmitted via the lateral surface.

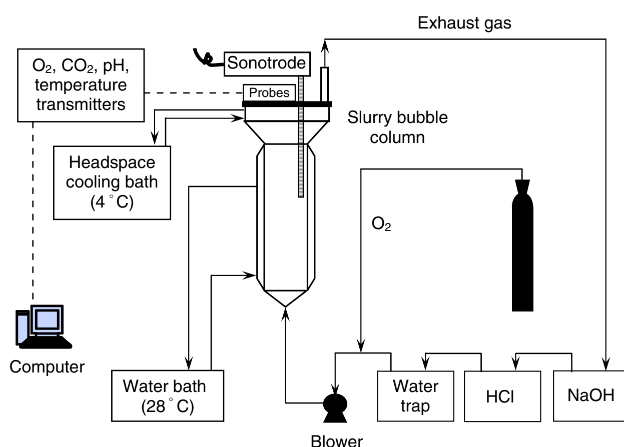

Fig. 1 Bubble column slurry sonobioreactor (Sainz Herrán et al. 2008)

In sonobioreactor experiments, cultures were sonicated continuously from 48-h after inoculation. The inoculum was a 48-h preculture grown as specified above to provide a consistent initial pellet morphology. Five continuous sonication energy levels were used. These were produced by various combinations of amplitude (*A*) and duration cycle pulse (*C*), as shown in Table 1.

Experimental design

A 2^k-factorial experimental design with three added central point replicates, was used. Seven experiments with different ultrasound intensities that combined several values of amplitude and cycle were carried out to assess the impact of sonication. In addition, a nonsonicated control culture was grown. Effects of the factors on the yield of lovastatin, biomass growth and morphology, and broth rheology, were assessed. The data were used to generate a response surface and for analysis of variance. Statgraphics™ software package (Manugistics Inc, Rockville, Maryland, USA) was used.

Concentration of fungal biomass

The fermentation broth contained biomass both in the form of pellets and as pulp-like free filamentous hyphae. Dry weight of these two forms was measured separately in each sample of the broth as specified previously (Sainz Herrán et al. 2008). Briefly, a known volume of the broth was sieved through a 2 mm sieve that retained mainly the fungal pellets. The broth that passed through the sieve was filtered through a 0.45 μm cellulose acetate membrane filter (Millipore) to recover the hyphae. The hyphal biomass was washed with sterile distilled water and freeze dried to a constant weight. The biomass pellets that remained on the sieve were resuspended in distilled water, filtered through a 0.45 μm cellulose acetate filter membrane, washed with sterile distilled water, and freeze dried to a constant weight.

Morphological characterization

Image analysis was used to characterize the fungal pellet morphology as discussed elsewhere (Paul and Thomas

Table 1 Sonication conditions

Run	Nomenclature	Amplitude level		Cycle level		Responses		Maximum sonic power input (W m^{-3})
		Coded	Real (%)	Coded	Real (%)	Biomass (g l^{-1})	Lovastatin (mg l^{-1})	
Control	(A0, C0)	−1.5	0	−1.5	0	9.78	223.31	0
1	(A20, C20)	−1	20	−1	20	9.75	194.92	111
2	(A60, C60) I	0	60	0	60	10.37	76.43	3,005
3	(A100, C100)	+1	100	+1	100	9.59	63.77	13,913
4	(A100, C20)	+1	100	−1	20	9.90	149.32	2,783
5	(A20, C100)	−1	20	+1	100	9.93	76.76	556
6	(A60, C60) II	0	60	0	60	9.89	97.34	3,005
7	(A60, C60) III	0	60	0	60	9.88	61.67	3,005

1998; Casas López et al. 2005; Rodríguez Porcel et al. 2005). For imaging, each sample of the fungal broth was prepared as follows: approximately 10 ml of sample was allowed to settle for a few minutes, the supernatant was discarded and the sedimented biomass was washed twice with 20 ml of distilled water. The solids were imaged using a CMOS camera (Evolution LC Color; Media Cybernetics, Inc., Silver Spring, MD, USA) installed on an inverted microscope (Leica DMIL; Leica Microsystems GmbH, Wetzlar, Germany) that provided a 40× magnification. Fifty objects were analyzed in each image for the various measurements. Image-Pro Plus 4.5.1 (Media Cybernetics, Inc., Silver Spring, MD, USA) software package was used for image analysis.

Pellets were characterized using the following two measures: (1) the diameter corresponding to a circular area equivalent to the total projected area of a pellet (Casas López et al. 2005); and (2) the ratio between the area of the peripheral "hairy surface" and the total projected area of a pellet (Casas López et al. 2005). The latter has been referred to as the "filament ratio" (Casas López et al. 2005). These two measures were indicative of the pellet size and the proportion of filamentous growth in it. For instance, at early stages of fermentation, a young pellet would typically have a small diameter and a filament ratio of close to 100%. As fermentation progressed, the filament ratio would reduce (Sainz Herrán et al. 2008; Casas López et al. 2005).

Broth rheology

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) was used to measure the flow behavior index n and the consistency index K (Sainz Herrán et al. 2008).

The jacketed viscometer vessel was held at 28°C during the measurements as previously described (Casas López et al. 2005). The glass vessel had a diameter of 35 mm and a fill depth of 70 mm. Calibration constants for the rotor vane were $c = 301.84$ and $k_i = 7.5994$ (Casas López et al. 2005).

Lovastatin concentration

Lovastatin in the broth existed in its β-hydroxy acid form and was measured as such by HPLC (Friedrich et al. 1995; Morovján et al. 1997; Sainz Herrán et al. 2008). Thus, a sample of the broth was filtered (0.45 µm cellulose acetate filter; Millipore). The filtrate was diluted tenfold with acetonitrile/water (1:1, v/v) and a 10 µl sample was injected in the HPLC (Shimadzu LC10 liquid chromatograph equipped with a Shimadzu MX-10AV diode array detector; Shimadzu Corp., Japan). A Hypersil Gold HPLC column (Thermo Fisher Scientific, Inc., Waltham, MA, USA; 150 × 4.6 mm I.D., 5 µm) was used at room temperature and a mobile phase flow rate of 1.25 ml min^{-1}. The mobile phase was a 50:50 v/v mixture of acetonitrile and 0.1% phosphoric acid. The detection wavelength was 238 nm.

Lovastatin standards were prepared using tablets (Nergadan® tablets; J. Uriach and Cía., S.A., Spain) of commercially available pharmaceutical-grade lovastatin (lactone form). The lactone form was first converted to the β-hydroxy acid 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. The standards with a precisely known concentration of lovastatin were filtered and analyzed in the same way as the samples.

Results and discussion

Fungal growth and morphology

The increase in biomass concentration with fermentation time is shown in Fig. 2 for variously sonicated

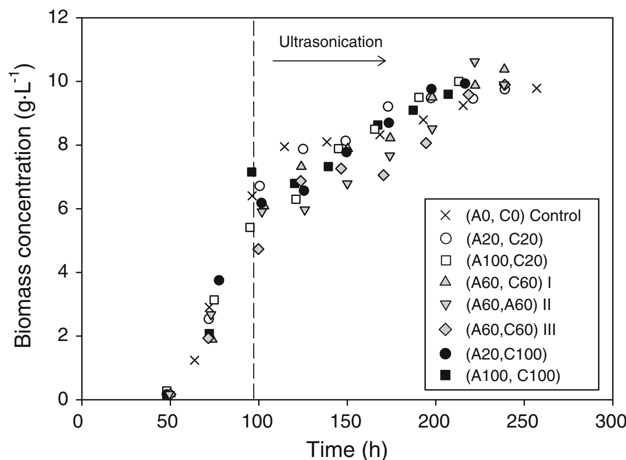

Fig. 2 Total biomass concentration versus fermentation time

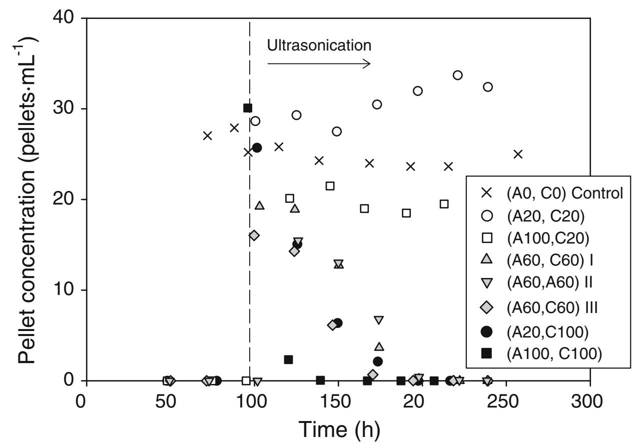

Fig. 3 Pellet concentration versus fermentation time

Table 2 Analysis of variance for the effects of ultrasound on final biomass concentration

Source	Sum of squares	*df*	Mean square	*F* ratio	*P* value
A: amplitude	0.009025	1	0.009025	0.12	0.7644
C: cycle	0.004225	1	0.004225	0.06	0.8364
AC	0.060025	1	0.060025	0.78	0.4699
Lack-of-fit	0.113668	1	0.113668	1.48	0.3479
Pure error	0.1536	2	0.0768		
Total (corr.)	0.340543	6			

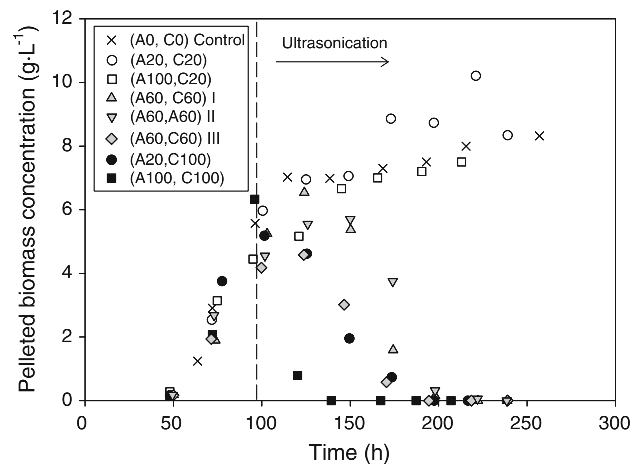

Fig. 4 Pellet biomass concentration versus fermentation time

fermentations and the control culture. In sonicated fermentations, ultrasound was applied from 96-h onwards. Sonication did not affect biomass growth rate and total biomass concentration at any combination of sonication amplitude (A) and duty cycle (C) (Fig. 2). The final biomass concentration was always around 10 g l^{-1} because of the quantity of nitrogen that was initially added to the medium. In concurrence with data reported previously for this fermentation (Casas López et al. 2003, 2004a, 2005; Sainz Herrán et al. 2008), the biomass yield on nitrogen was always around 25 g g^{-1}. Analysis of variance in Table 2 for the effect of sonication on the final biomass concentration confirms that sonication amplitude and cycle did not affect the final concentration (i.e. P-values greater than 0.05) at the 95% confidence level. Similarly, there were no interactive effects of sonication amplitude and cycle on the final biomass concentration (Table 2).

Concentration of pellets (i.e. number of pellets ml^{-1}) and pelleted biomass (g l^{-1}) varied during the fermentation as shown in Figs. 3 and 4, respectively. Both the concentrations of pellets and the pelleted biomass were affected by sonication at ≥60% cycle, irrespective of the value of the amplitude (Figs. 3, 4). Sonication at ≥60% cycle caused a progressive decline in the concentration of pellets (Fig. 3) and pelleted biomass (Fig. 4) from the instance at which sonication commenced (i.e. 96-h). In general, the effect of the sonication cycle became more pronounced as the value of the sonication cycle increased above 60%. Sonication cycle of ≤20% did not affect concentrations of pellets (Fig. 3) and pelleted biomass (Fig. 4), irrespective of the amplitude of operation.

As the total concentration of biomass (i.e. pelleted biomass and freely suspended hyphal biomass) was unaffected by sonication (Fig. 2) at any intensity, a reduced concentration of pelleted biomass (Fig. 4) with sonication necessarily implied a corresponding increase in the amount of freely suspended hyphal biomass. This was indeed confirmed by measurements of the fraction of hyphal biomass in the fermentation broth, as shown in Fig. 5. Sonication at cycle values of ≥60% increased the fraction of dispersed hyphal biomass (Fig. 5). Thus, clearly, sonication at cycle values of ≥60% fragmented the pellets to produce more free hyphal biomass, but did not reduce biomass viability as the growth rate was not affected

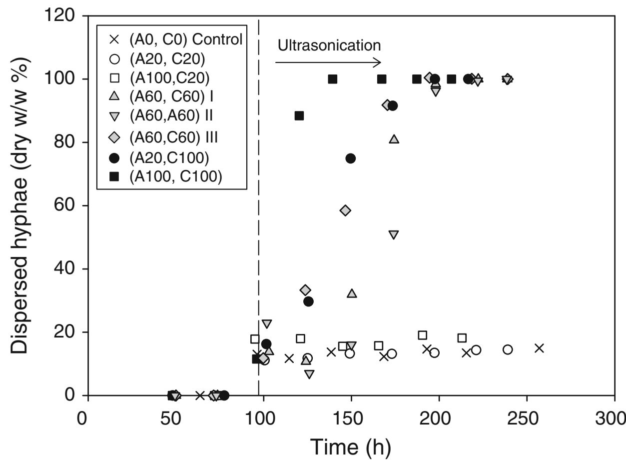

Fig. 5 Fraction of dispersed hyphae (dry w/w %) in the broth versus fermentation time

(Fig. 2). This occurred irrespective of the value of the sonication amplitude. In cultures that were sonicated at cycle times of ≥60%, all the biomass eventually attained a freely suspended hyphal morphology (Fig. 5).

An analysis of variance (ANOVA) of the data in Fig. 5 is shown in Table 3 at different times during the fermentation. At any time during the fermentation, the sonication cycle was clearly the factor that had the greatest influence on the fraction of free hyphal biomass in the broth (*P*-values mostly <0.1). For the most part, sonication amplitude did not have a great impact on the fraction of hyphal biomass (Table 3; *P*-values mostly >0.1). Furthermore, in nearly all cases, there was barely any interactive effect of amplitude and cycle on the fraction of the hyphal biomass present.

The biomass produced in submerged fermentations by *A. terreus* germinated from spores, occurred as pellets, or small dense clumps of hyphae, and freely suspended hyphae. All the fermentations in this work initially had an overwhelmingly pelleted biomass morphology (Fig. 5; ≤96-h). Turbulent attrition of pelleted biomass is known to erode surface hyphae and reduce dimensions of pellets especially after growth has ceased (Casas López et al. 2005). This gives rise to an increasing fraction of free

Table 3 Analysis of variance of the effect of sonication on percentage of free hyphae at various times during fermentation

Time (h)	*A*: Amplitude		*C*: Cycle		*AC* interaction	
	F ratio	*P* value	*F* ratio	*P* value	*F* ratio	*P* value
100	0.03	0.8755	0.01	0.9229	1.04	0.4151
125	5.19	0.1503	9.63	0.09	3.40	0.2066
150	0.41	0.5877	11.62	0.0763	0.28	0.6483
175	0.15	0.7330	14.4	0.063	0	0.9938
200	14.24	0.0636	1821.75	0.0005	14.24	0.0636

hyphae (Fig. 5). Compared with control culture, however, the fraction of dispersed hyphae increased much more rapidly with time in fermentations that were sonicated at cycle values of ≥60% (Fig. 5). In these sonicated fermentations, ultrasound contributed substantially to generation of free hyphae not so much through erosion of pellets, but by their sudden fragmentation. Furthermore, the free hyphae produced were generally shorter and less branched compared with the free hyphae that occurred in nonsonicated control culture. Reduced length and branching of dispersed hyphae resulted in a less viscous broth compared with the control culture, as discussed in a later section of this paper.

The morphology of fungal solids was characterized in terms of the mean diameter of the pellets and their filament ratio (Casas López et al. 2005). The measured average diameter of fungal pellets was roughly 1,500 µm at inoculation (Fig. 6a). During the next 48 h, the pellets increased in size to around 2,900 µm as a consequence of growth. Afterwards, the pellets decreased in size somewhat because the growth had slowed (Fig. 2) and, therefore, the

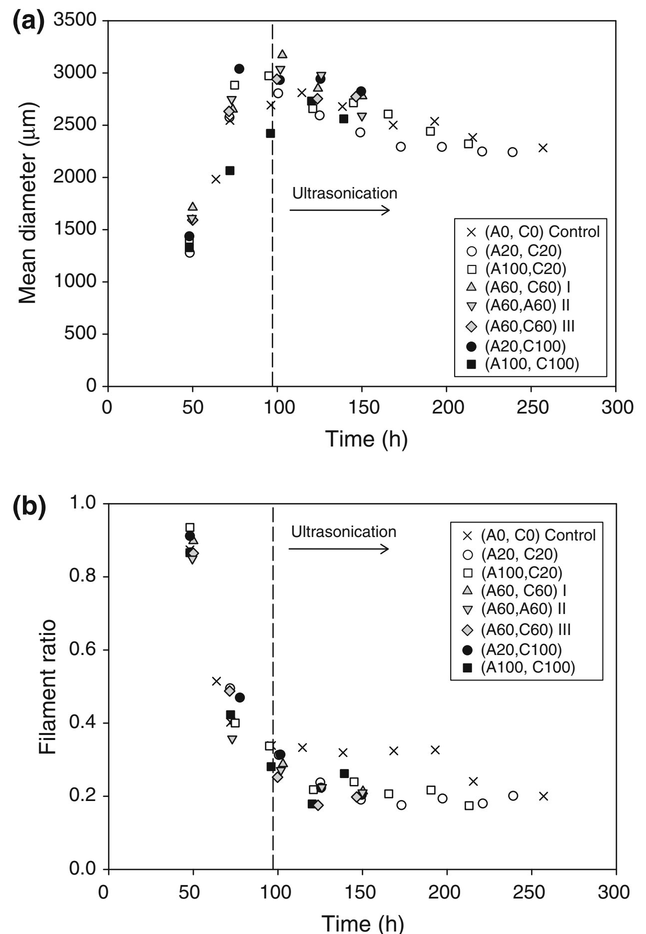

Fig. 6 Mean pellet diameter (**a**) and mean filament ratio (**b**) versus fermentation time

rate of pellet erosion due to turbulence in the broth had exceeded the rate of pellet growth. Sonication at ≥60% cycle did not have much effect on diameter and filament ratio of the surviving pellets in comparison with the control culture (Fig. 6), but it did greatly reduce the concentration of the pellets (Fig. 3).

In nonsonicated stirred tank bioreactors, mechanical agitation is known to affect the morphology of *A. terreus* pellets (Casas López et al. 2005; Rodríguez Porcel et al. 2005). The mean pellet diameter declines with increasing total power input, but stable pellets of roughly 800 µm in diameter persist at power input values that are as high as 2,000 W m^{-3} (Casas López et al. 2005; Rodríguez Porcel et al. 2005). In contrast, in the slurry bubble column reactor at 20% sonication amplitude, 100% sonication cycle and 1 vvm of air flow rate, the pellets did not survive even though the total power input was only about 769 W m^{-3}, or much lower than in the stirred tank in which the pellets had survived. Despite this relatively low power input, pellets were ruptured spontaneously in the sonicated bioreactor. At ≥60% sonication cycle, no pellets survived for long even though the viability of the biomass was not affected. This was likely because the local energy dissipation rate in the vicinity of the sonotrode tip was substantially greater than the calculated average power input. Mechanism of pellet rupture in the sonobioreactor was clearly quite different from that in a stirred tank.

Power input in the slurry bubble column (no ultrasound) was calculated as the power imparted to the fluid by isothermal expansion of the gas as described in the "Materials and methods" section. This power input was 213 W m^{-3}. During sonication, the additional power input was directly proportional to the cycle value and to the square of the amplitude. The maximum sonication power that could be provided with the equipment employed was around 13,913 W m^{-3} (Table 1).

As shown in Fig. 6b, the filament ratio declined with time from an initial value of around 90% to approximately 33% (at 150-h) in the control culture (no ultrasound) in agreement with earlier observations (Casas López et al. 2005; Rodríguez Porcel et al. 2007). For sonicated broths, the final values of the filament ratio were around 20% at 150-h.

Rheology of fungal broth

Rheology, or the flow behavior, of a fungal fermentation broth depends on the concentration and morphology of the biomass. A broth with a high proportion of interacting freely suspended hyphae is generally more viscous compared to one containing the same total amount of biomass in the form of pellets. *A. terreus* broth rheology is commonly characterized in terms of a flow behavior index n and a consistency index K (Casas López et al. 2005; Rodríguez Porcel et al. 2005, 2006; Sainz Herrán et al. 2008). The values of K and n are shown in Fig. 7a and b, respectively, at different times during the fermentations.

For the sonicated fermentations as well as the nonsonicated control, the K-value declined progressively with fermentation time after the first 96-h (Fig. 7a) even though there was barely any biomass growth during the same period (Fig. 2), as explained earlier. The fraction of the biomass present as dispersed hyphae did not vary much after the first 96-h in the control fermentation and the fermentation conducted at the low sonication cycle of 20% (Fig. 5).

Sonication at cycle values greater than 60%, rapidly elevated the fraction of dispersed hyphae soon after sonication began (Fig. 5), but the K-value continued to decline (Fig. 7a). This was contrary to expectations because in many fungal fermentations, the K-value increases as the fraction of dispersed hyphae increases (Sainz Herrán et al. 2008). The reason for the unexpected behavior was that the free hyphae produced by the ultrasound mediated rupture of pellets were unbranched and smaller than the hyphae

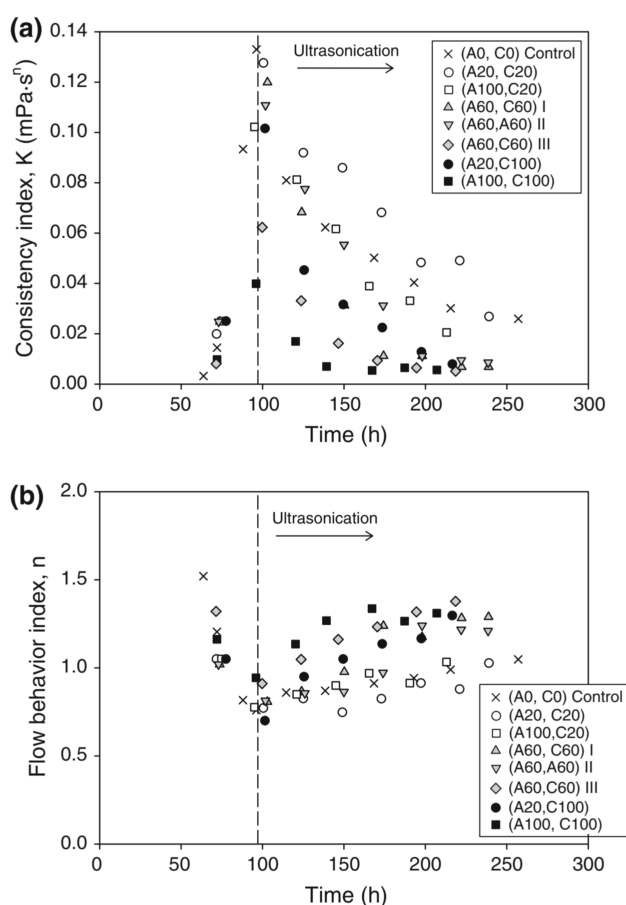

Fig. 7 Power law parameters versus fermentation time: **a** consistency index, K; **b** flow behavior index, n

that would be produced normally in fungal growth (Sainz Herrán et al. 2008). Concentration of pellets declined with increasing duration of sonication (Fig. 4), as discussed earlier.

A high proportion of the biomass in the control fermentation formed pellets, but some biomass remained in the form of free filaments or small fluffy clumps. The proportion of pellets to freely suspended hyphae varied with time during the fermentation. Around 100-h of cultivation, the pelleted growth represented about 85% of the biomass and the pellet diameter attained its maximum value. However, the small fraction of the biomass that was present as freely suspended branched hyphae caused extensive bridging of pellets by entangling with the hyphae that extended from the surfaces of the pellets. This entanglement of pellets made the broth highly viscous. A similar behavior was seen in fermentations that were not intensely sonicated (Fig. 5). As the fermentation progressed nitrogen became limiting. This not only prevented further growth of the hyphae but actually caused some lysis of the free filaments and a reduction in size of the pellets. Consequently, around 200-h, pellet–pellet interactions reduced and this greatly reduced the *K*-value of the broth (Fig. 7a).

As expected, for the first 96-h when none of the fermentations was sonicated, the *n*-values were similar for all broths (Fig. 7b). In all cases, the initial value of *n* was relatively high but declined to around 0.7 by 100-h. Thus, the fluids became pseudoplastic from being initially dilatant (Fig. 7b). This was a consequence of an increasing biomass concentration (Fig. 2), an increasing average pellet diameter (Fig. 6a), and a decreasing filament ratio of the pellets (Fig. 6b).

From 96-h until the end of the fermentation, the *n*-values gradually increased with time. The rate of increase was higher relative to control, in cultures sonicated at cycle times of ≥60% (Fig. 7b). Clearly, therefore, the broth rheology was affected by sonication at cycle values of greater than 60%, but the exact cause of this is not clear. Sonication may have reduced the tendency of the hyphae to entangle by shortening them.

Lovastatin

Production profiles of lovastatin are shown in Fig. 8. Production was severely and negatively affected by sonication when the cycle values exceeded 20%. As discussed above, sonication did not influence the biomass growth rate and concentration. This suggests that ultrasound influenced production of lovastatin, a secondary metabolite, without affecting the primary growth metabolism. Earlier studies have shown that in *A. terreus* lovastatin is produced preferentially during pelleted growth (Casas López et al. 2005; Rodríguez Porcel et al. 2005), as a probable consequence of the microenvironment afforded by the pellets. Once the sonication cycle values exceeded 20%, ultrasound caused a rupture of the pellets and this could have indirectly affected the synthesis of lovastatin. A comparison of Figs. 4 and 8 confirms that the fermentations that retained a pelleted morphology continued to produce lovastatin.

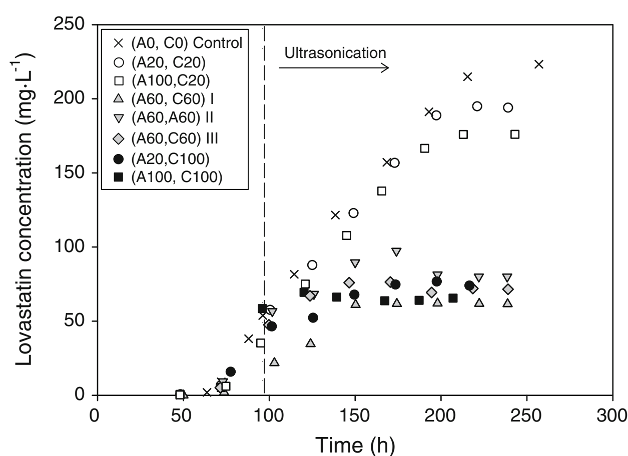

Fig. 8 Lovastatin concentration versus fermentation time

An analysis of variance for the effects of sonication on the final concentration of lovastatin in the fermentation broth is shown in Table 4. The factor that had the most effect on final lovastatin concentration was the sonication cycle value (i.e. a *P*-value of less than 0.05).

Since the *P*-value for the lack-of-fit for a first order model was low and near to 0.05, a second order model was developed to consider the second-order effects and to explain the influence of the factors on the final concentration of lovastatin (Table 4). The following second-order polynomial best fitted the data:

$$\text{Lovastatin (mg l}^{-1}) = 2.364 \times 10^2 - 2.718 \times 10^{-1}A - 3.418C + 4.790 \times 10^{-4}AC + 1.866 \times 10^{-2}C^2 \quad (2)$$

where *A* and *C* are sonication amplitude and cycle values, respectively. The r^2-value for the fit explained 94.5% of the variability in the final lovastatin concentration.

Table 4 Analysis of variance for effects of sonication on final lovastatin concentration

Source	Sum of squares	*df*	Mean square	*F*-ratio	*P* value
A: amplitude	424.284	1	424.284	1.32	0.3693
C: cycle	9,497.17	1	9,497.17	29.56	0.0322
AC	3.11033	1	3.11033	0.01	0.9306
C^2	2,238.77	1	2,238.77	6.97	0.1185
Lack-of-fit	892.609	1	892.609	2.78	0.2375
Pure error	642.478	2	321.239		
Total (corr.)	27,957.4	7			

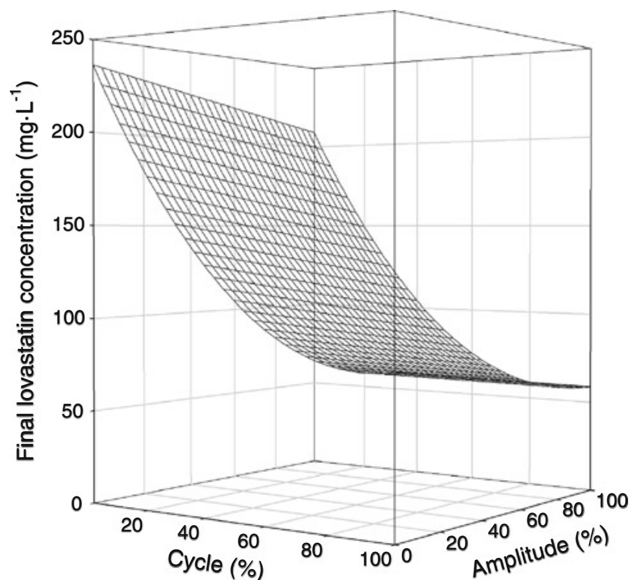

Fig. 9 Estimated response surface for lovastatin concentration for various values of sonication amplitude and cycle

The response surface generated by Eq. 2 is shown in Fig. 9. Equation 2 predicts a final lovastatin concentration in the absence of sonication, of 236.4 mg l^{-1}, quite close to the observed value of 223.3 mg l^{-1} in the control culture. Figure 9 further reveals that sonication cycle value has a far greater influence on the final lovastatin concentration than does the sonication amplitude. In addition, the second order dependence of the final lovastatin concentration on the cycle value implies a saturation effects of the cycle on the final concentration for cycle values of higher than $\sim$ 80%.

Conclusions

Ultrasound is potentially useful for influencing metabolite production, growth morphology, broth rheology and the associated transport phenomena in fermentations of filamentous fungi. These effects can be achieved without damaging the microorganism. Growth rate and biomass yield of *A. terreus* were not affected by continuous sonication at any of the power intensities tested; however, fungal morphology was profoundly affected by sonication at cycle values $\geq$60%. Whereas the nonsonicated control fermentation was characterized predominantly by a pelleted growth of the biomass, this morphology was entirely replaced by dispersed hyphal growth by sonication at 100% cycle value. Sonication reduced production of lovastatin. No measureable effects of ultrasound on fungal morphology were seen below sonication cycle values of 60%. Consideration of such effects may be important in other industrial fermentations.

Acknowledgments This research was supported by the Ministerio de Educación y Ciencia (Grant CTQ2004-04454/PPQ) and Junta de Andalucía (Grant BIO 263), Spain.

References

Casas López JL, Sánchez Pérez JA, Fernández Sevilla JM, Acién Fernández FG, Molina Grima E, Chisti Y (2003) Production of lovastatin by *Aspergillus terreus*: effects of the C:N ratio and the principal nutrients on growth and metabolite production. Enzyme Microb Technol 33:270–277

Casas López JL, Rodríguez Porcel EM, Vilches Ferrón MA, Sánchez Pérez JA, Fernández Sevilla JM, Chisti Y (2004a) Lovastatin inhibits its own synthesis in *Aspergillus terreus*. J Ind Microbiol Biotechnol 31:48–50

Casas López JL, Sánchez Pérez JA, Fernández Sevilla JM, Acién Fernández FG, Molina Grima E, Chisti Y (2004b) Fermentation optimization for the production of lovastatin by *Aspergillus terreus*: use of the response surface methodology. J Chem Technol Biotechnol 79:1119–1126

Casas López JL, Sánchez Pérez JA, Fernández Sevilla JM, Rodríguez Porcel EM, Chisti Y (2005) Pellet morphology, culture rheology and lovastatin production in cultures of *Aspergillus terreus*. J Biotechnol 16:61–77

Chisti Y (1989) Airlift bioreactors. Elsevier, London, p 350

Chisti Y (1999) Mass transfer. In: Flickinger MC, Drew SW (eds) Encyclopedia of bioprocess technology: fermentation, biocatalysis and bioseparation, vol 3. Wiley, New York, pp 1607–1640

Chisti Y (2003) Sonobioreactors: using ultrasound for enhanced microbial productivity. Trends Biotechnol 21:89–93

Chisti Y, Moo-Young M (1986) Disruption of microbial cells for intracellular products. Enzyme Microb Technol 8:194–204

Friedrich J, Zuzek M, Bencina M, Cimerman A, Strancar A, Radez I (1995) High-performance liquid chromatographic analysis of mevinolin as mevinolinic acid in fermentation broths. J Chromatogr A 704:363–367

Grothe E, Moo-Young M, Chisti Y (1999) Fermentation optimization for the production of poly (β-hydroxybutyric acid) microbial thermoplastic. Enzyme Microb Technol 25:132–141

Hajjaj H, Niederberger P, Duboc P (2001) Lovastatin biosynthesis by *Aspergillus terreus* in a chemically defined medium. Appl Environ Microbiol 67:2596–2602

Kumar MS, Jana SK, Senthil V, Shashanka V, Kumar SV, Sadhukhan AK (2000) Repeated fed-batch process for improving lovastatin production. Process Biochem 36:363–368

Lai LST, Pan CC, Tzeng BK (2003) The influence of medium design on lovastatin production and pellet formation with a high-producing mutant of *Aspergillus terreus* in submerged cultures. Process Biochem 38:1317–1326

Manzoni M, Rollini M, Bergomi S, Cavazzoni V (1998) Production and purification of statins from *Aspergillus terreus* strains. Biotechnol Tech 12:529–532

Manzoni M, Bergomi S, Rollini M, Cavazzoni V (1999) Production of statins by filamentous fungi. Biotechnol Lett 21:253–257

Morovján G, Szakács G, Fekete J (1997) Monitoring of selected metabolites and biotransformation products from fermentation broths by high-performance liquid chromatography. J Chromatogr A 763:165–172

Novak N, Gerdin S, Berovic M (1997) Increased lovastatin formation by *Aspergillus terreus* using repeated fed-batch process. Biotechnol Lett 19:947–948

Paul GC, Thomas CR (1998) Characterisation of mycelial morphology using image analysis. Adv Biochem Eng 60:1–59

Rodríguez Porcel EM, Casas López JL, Sánchez Pérez JA, Fernández Sevilla JM, Chisti Y (2005) Effects of pellet morphology on broth rheology in fermentations of *Aspergillus terreus*. Biochem Eng J 26:139–144

Rodríguez Porcel EM, Casas López JL, Sánchez Pérez JA, Fernández Sevilla JM, García Sánchez JL, Chisti Y (2006) *Aspergillus terreus* broth rheology, oxygen transfer and lovastatin production in a gas-agitated slurry reactor. Ind Eng Chem Res 45:4837–4843

Rodríguez Porcel EM, Casas López JL, Sánchez Pérez JA, Chisti Y (2007) Enhanced production of lovastatin in a bubble column by *Aspergillus terreus* using a two-stage feeding strategy. J Chem Technol Biotechnol 82:58–64

Sainz Herrán N, Casas López JL, Sánchez Pérez JA, Chisti Y (2008) Effects of ultrasound on culture of *Aspergillus terreus*. J Chem Technol Biotechnol 83:593–600

Sánchez Pérez JA, Rodríguez Porcel EM, Casas López JL, Fernández Sevilla JM, Chisti Y (2006) Shear rate in stirred tank and bubble column bioreactors. Chem Eng J 124:1–5

Szakács G, Morovján G, Tengerdy RP (1998) Production of lovastatin by a wild strain of *Aspergillus terreus*. Biotechnol Lett 20:411–415