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Effects of ultrasound on culture of *Aspergillus terreus*

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Abstract

BACKGROUND: Fermentations of *Aspergillus terreus* are commercially used to produce lovastatin. How ultrasound might influence this fermentation is unknown. While high-intensity ultrasound is effective in disrupting microbial cells, ultrasound of low intensity is known to improve productivity of some fermentation processes without damaging cells. Mechanisms behind productivity improvements have not been clearly identified in earlier studies. This work reports on the effects of ultrasound on *A. terreus* fermentation for low (957 W m^{-3}), medium (2870 W m^{-3}) and high (4783 W m^{-3}) values of sonication power input in a slurry bubble column sonobioreactor.

RESULTS: Sonication at any power level did not affect biomass growth profiles in comparison with negative controls. In contrast, medium- and high-intensity sonication greatly reduced production of lovastatin and substantially altered the growth morphology. At medium and high intensity, ultrasound disrupted fungal pellets and caused the biomass to grow mainly as dispersed hyphae. Sonication affected broth rheology because rheology depends on the morphology of the suspended biomass.

CONCLUSION: Sonication can be used to modify growth morphology and broth rheology without affecting growth of filamentous fungi. Sonication appears to influence the primary growth metabolism and secondary metabolism differently in different situations.
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Keywords: sonobioreactors; ultrasound; lovastatin; *Aspergillus terreus*; fungal morphology; fungal rheology

INTRODUCTION

Ultrasound, or sound of frequency >20 kHz, is inaudible to the human ear. Ultrasonication of microbial suspensions is routinely used to break cells for releasing intracellular products.[1] Although high-intensity ultrasound is extremely effective in breaking microbial cells, ultrasound of low intensity is known to improve productivity of at least some fermentation processes without damaging cells.[2] Mechanisms behind productivity improvements have not been clearly identified, although factors such as improved gas–liquid mass transfer and solid–liquid mass transfer may play a role. Ultrasound may also alter metabolic processes within an otherwise healthy cell. This work reports on the effects of ultrasound on *Aspergillus terreus* batch fermentations.

Ultrasound is produced using magneto-restrictive or piezoelectric transducers, which convert the alternating current of an electronic oscillator to mechanical waves that are transmitted to the broth through a cylindrical rod-shaped probe, or sonotrode.[2] Bioreactors designed for sonicated culture of cells are known as sonobioreactors.[2] Most of the effects of sonication are linked with the energy it imparts to the culture broth.[2] Sonic energy is mostly dissipated at the tip of the sonotrode. During the rarefaction phase of the sound wave, the low pressure developed at the tip causes cavitation microbubbles to form and grow. During the compression phase, the bubbles implode, releasing a violent shock wave that propagates through the broth.[2]

Fermentations of the filamentous fungus *A. terreus* are commercially used to produce lovastatin, a potent drug for lowering blood cholesterol. Pelleted growth of *A. terreus* is known to yield higher titers of lovastatin compared with the titers obtained in dispersed filamentous growth.[3–5] Fungal morphology has been shown to be sensitive to the intensity of hydrodynamic and mechanical shear forces in bioreactors.[4–6] For identical concentrations of biomass, a broth with dispersed filamentous growth is highly viscous compared with a broth with predominantly pelleted

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growth. Viscous broths impede oxygen transfer and this is said to explain the low titers of lovastatin during dispersed filamentous growth.[3] Although lovastatin production by *A. terreus* has been extensively investigated,[3–5,7–17] how ultrasound might influence this fermentation is unknown. Some of the published information[4,13–15] provided a basis for the fermentation conditions used in the present study. Effects of ultrasound on other fungal fermentations have been reported.[18–21] Use of ultrasound in influencing fermentations has been reviewed elsewhere.[2]

MATERIALS AND METHODS

Microorganism and inoculation

A. terreus ATCC 20 542 was obtained from the American Type Culture Collection. The fungus was maintained in Petri dishes of potato dextrose agar (PDA). 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 ($5000 \times g$, 3 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; Beckman Coulter Ltd, High Wycombe, UK).

Growth conditions

Fungal pellets were obtained by germination from spores suspended in shake flasks in a preliminary fermentation stage, and used for further inoculation of the slurry bubble column bioreactor. Seed cultures were carried out in 1000 mL flasks containing 300 mL of medium, held on a rotary shaker at 150 rpm, 28 °C, for 48 h. For all fermentations, 850 mL of seed culture was used to inoculate the bioreactor.

The culture medium contained lactose as carbon source and soybean meal as nitrogen source. The medium contained per liter: 114.26 g lactose, 5.41 g soybean meal, 0.8 g KH_2PO_4, 0.4 g NaCl, 0.52 g $MgSO_4.7H_2O$, 1 mg $ZnSO_4.H_2O$, 2 mg $Fe(NO_3)_3.9H_2O$, 0.04 mg biotin and 1 mL of a trace element solution. The trace element solution contained (for 1 L of solution): $Na_2B_4O_7.10H_2O$, 100 mg; $MnCl_2.4H_2O$, 50 mg; $Na_2MoO_4.2H_2O$, 50 mg; and $CuSO_4.5H_2O$, 250 mg. The initial pH was adjusted to 6 with 0.1 mol L^{-1} NaOH. The medium used here had been previously optimized for lovastatin production by the same fungus.[14]

Bubble column fermentations

Fermentations were carried out at 28 °C in a slurry bubble column reactor (Fig. 1) of 25 L total volume (23 L working volume). The diameter of the reactor vessel was 0.15 m and the aspect ratio was 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 was fitted with a jacket 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. Each fermentation lasted 8 days.

The dissolved oxygen concentration in the liquid was controlled at 400% of air saturation by supplementing the gas phase with pure oxygen. The gas leaving the reactor was recirculated in a closed loop. Prior to recirculation, any carbon dioxide was removed by absorption in an NaOH solution in order to keep the CO_2 concentration in the sparged gas below 0.1% v/v. Pure oxygen was added to the gas phase to maintain the specified dissolved oxygen level in the liquid. Dissolved oxygen was measured online in the liquid phase using a polarographic electrode (model InPro

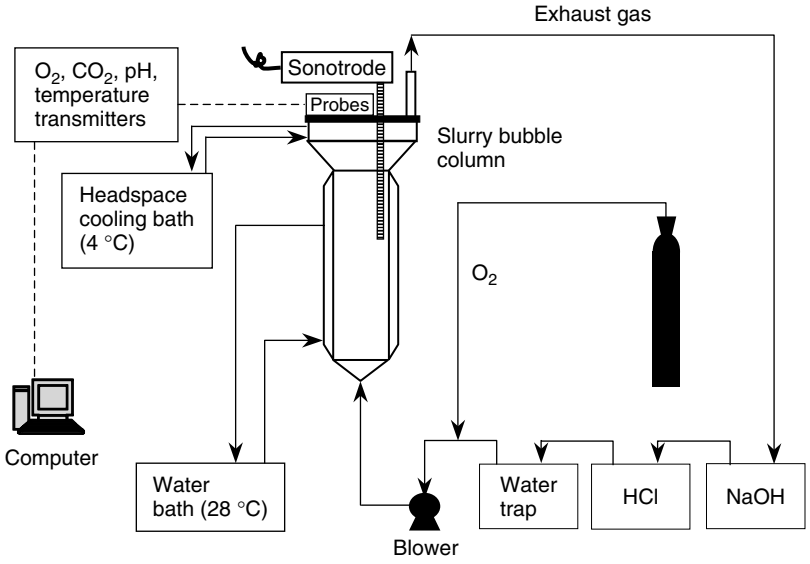

Figure 1. Slurry bubble column sonobioreactor.

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6100/220T; Mettler-Toledo, Inc., Columbus, OH, USA). The dissolved oxygen electrode was calibrated in the sterilized culture medium. For this, the liquid was bubbled with air and the concentration of oxygen was set at a saturation value of 100%. In view of the generally turbulent and steady hydrodynamic conditions during measurements, any effects of the liquid film at the surface of the electrode were disregarded, as is typical for this kind of work.[22] Power input due to isothermal expansion of the aeration gas was 213 W m^{-3}.

Sonobioreactor fermentations

For use as sonobioreactor, the slurry bubble column described earlier was installed with a titanium sonotrode (22 mm in diameter, 300 mm long, model H22L3D; Hielscher GmbH, Stuttgart, Germany) connected to a generator (model UP400S, Hielscher). The sonotrode was inserted in the reactor column at the headplate (Fig. 1). The power level could be varied by adjusting the amplitude and cycle time of the sonotrode between 20% and 100%. The amplitude is directly related to power input. A cycle time of 20%, for example, is equal to ultrasound being applied for 0.2 s every second. The sonotrode could deliver a maximum of 320 W power. Of this, 120 W was transmitted through the lateral surface and 200 W was transmitted from the tip.

All the cultures were sonicated continuously from 48 h after inoculation. The inoculum was a 48 h pre-culture grown in a 1 L shake flask to provide a consistent initial pellet morphology. Three energy levels of continuous ultrasound were used. These were obtained by various combinations of amplitude (A) and duty cycle (C). The lowest energy input of 957 W m^{-3} was obtained using an amplitude of 20% and a 20% cycle. A 60%–60% A–C combination delivered 2870 W m^{-3} to the culture bulk. The upper power input value used was 4783 W m^{-3}. This was attained using an A–C combination of 100%–100%. For experiments with sonication, the power input due to ultrasound ranged from 86% to 97% of the total power input. The rest of the power was contributed by isothermal expansion of the aeration gas, as the only other source of power present. Power input due to isothermal expansion was 213 W m^{-3}, as estimated from the measured aeration rate.[22]

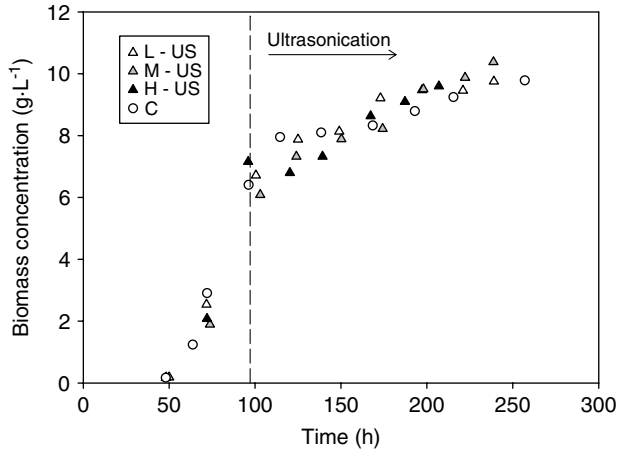

Figure 2. Total biomass concentration *versus* fermentation time. L-US: low sonication intensity; M-US: medium sonication intensity; H-US: high sonication intensity; C: control (no sonication).

Experimental data presented here are mean values of two replicates. The following notation is used to describe the various culture conditions: L-US for sonication involving an amplitude of 20% with a 20% cycle; M-US for sonication involving an amplitude of 60% with a 60% cycle; H-US for 100% amplitude and 100% cycle and C for control culture, which had the same bioreactor configuration as the sonicated cultures but without the ultrasound being applied.

Biomass concentration

The biomass in the fermentation broth was present partly as free filamentous hyphae and partly as pellets. These two morphological forms were quantified separately in a known volume of the broth sample. A sample of the broth was sieved through a 2 mm sieve that retained mostly the pellets. The sample that passed through the sieve was filtered through a 0.45 µm Millipore cellulose filter that retained the hyphae. The hyphal solids were washed with sterile distilled water and freeze dried to constant weight. The biomass pellets recovered on the sieve were resuspended in distilled water, filtered through a 0.45 µm cellulose filter, washed with sterile distilled water, and freeze dried to constant weight.

Morphological measurements

Fungal pellet morphology was characterized using image analysis.[4,5,23] Prior to imaging, each sample of the fermentation broth was processed as follows: approximately 10 mL of sample was decanted and washed twice with 20 mL of distilled water. Within a sample, 50 objects were analyzed for each determination. The image was captured with a CMOS camera (Evolution LC Color; Media Cybernetics, Inc., Silver Spring, MD, USA) mounted on an inverted microscope (Leica DMIL; Leica Microsystems GmbH, Wetzlar, Germany) that used a ×40 magnification. Image analysis was performed with the software package Image-Pro Plus 4.5.1 (Media Cybernetics).

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;[4] and (2) the ratio between the area of the peripheral 'hairy surface' and the total projected area of the pellet.[4] This ratio was termed the 'filament ratio'.[4] These two measures provided a direct indication of the pellet size and the proportion of filamentous growth in it. For instance, in the early stages of fermentation, a young pellet would be typically characterized by a small diameter and a filament ratio close to 100%. As fermentation progressed, the filament ratio would reduce.

Rheological measurements

Rheological power law 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 in a jacketed glass vessel at 28 °C as described by Casas López *et al.*[4] The glass vessel had a diameter of 35 mm and was filled to 70 mm. Calibration constants for the rotor vane were $c = 301.84$ and $k_i = 7.5994$.[4]

Lovastatin concentration

Lovastatin was measured in its β-hydroxy acid form by HPLC of the biomass-free filtered broth.[24,25] The filtered broth containing the β-hydroxy acid 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, Barcelona, Spain) were used to prepare the standards for HPLC analyses. Prior to use, the lactone form was converted into its β-hydroxy acid form by dissolving the tablets in a mixture of 0.1 N NaOH and ethanol (1:1, v/v), heating the solution at 50 °C for 20 min, and neutralizing it with HCl.

HPLC analysis was done on a Shimadzu LC10 liquid chromatograph equipped with a Shimadzu MX-10AV diode array detector (Shimadzu Corp., Kyoto, Japan). A Hypersil Gold HPLC column (Thermo Fisher Scientific, Inc., Waltham, MA, USA; 150 × 4.6 mm i.d., 5 μm) was used. The eluent was a 50:50 v/v mixture of acetonitrile and 0.1% phosphoric acid. The eluent flow rate was 1.25 mL min^{-1}. The detection wavelength was 238 nm. The sample injection volume was 20 μL.

RESULTS AND DISCUSSION

Biomass growth and morphology

Figure 2 shows the biomass growth curves during fermentation for the three ultrasonication regimens (low (L-US), medium (M-US) and high (H-US)) and for the control culture (i.e., no sonication). All the growth profiles are seen to have the same behavior (Fig. 2), leading to the conclusion that the biomass growth is not affected by sonication at any intensity (Fig. 2). In all cases, the final biomass concentration depended solely on the availability of the limiting nutrient, in this case, the nitrogen source. The mean final biomass concentration of around 10 g L^{-1} was equivalent to a biomass yield on nitrogen of around 25 g g^{-1}, or quite consistent with previously reported data for this fermentation.[4,13,14]

In all cases, the measured average fungal pellet diameter at inoculation was roughly 1500 μm (Fig. 3a). As growth occurred in the following 48 h, the pellets increased in size to around 2800 μm. Subsequently, the pellet diameter declined somewhat because the growth had slowed (Fig. 2) and, therefore, the rate of pellet growth became less than the rate of pellet erosion due to turbulence in the broth. The final stable pellet diameter was around 2500 μm (Fig. 3a), or quite consistent with earlier observations.[5,16,17] In the culture sonicated at high intensity (i.e. H-US, Fig. 3a), pellets ceased to exist from 150 h onwards because of ultrasound-induced breakage into clumps and hyphae. In broth sonicated at lower intensity and non-sonicated control culture, the pellets survived in the reactor until the end of the fermentation but the mean pellet diameter decreased because of hydrodynamic erosion in the absence of significant biomass growth (Fig. 2).

Filament ratio declined with time from an initial value of around 90% to approximately 35% in control culture (no ultrasound) (Fig. 3b), in agreement with earlier observations.[4,17] For sonicated broths, the final values of the filament ratio were significantly lower at 25% than in control culture (Fig. 3b). This occurred irrespective of the sonication regimen. Sonication enhanced compactness of pellets by inducing loss of the outer hyphal layer.

Although pellet diameter was marginally affected by ultrasound, sonication at medium and high intensities had a substantial effect on the fraction of dispersed hyphae in the broth (Fig. 4). The fraction of dispersed hyphae in low-level sonicated broth was comparable

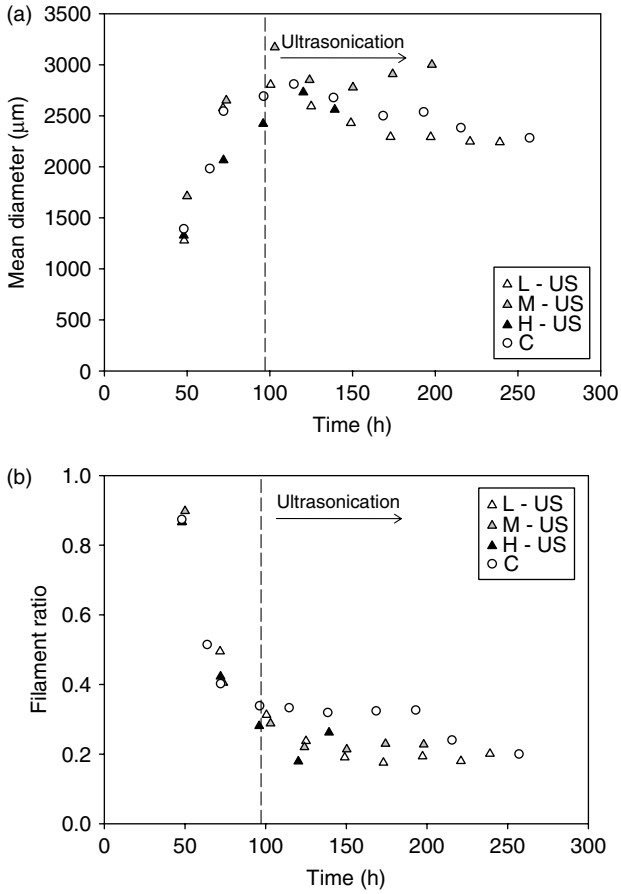

Figure 3. Mean pellet diameter (a) and mean filament ratio (b) *versus* fermentation time. L-US: low sonication intensity; M-US: medium sonication intensity; H-US: high sonication intensity; C: control (no sonication).

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to that of the control culture for the entire duration of fermentations (Fig. 4). Sonication intensity further affected the rate at which the dispersed hyphae were generated (Fig. 4). At medium and high sonication intensities, most of the biomass in the broth eventually existed in the form of dispersed hyphae (Fig. 4).

Because the total biomass concentration was unaffected by sonication (Fig. 2), a high fraction of hyphal biomass in cultures sonicated at high and medium intensities necessarily implied a reduced concentration of the pelleted biomass in these cultures. This was consistent with the measurements of pellet concentrations, as shown in Fig. 5. Clearly, the pellet concentration declined rapidly for medium- and high-intensity sonication as soon as sonication commenced (Fig. 5). The rate of decline in concentration of pelleted biomass increased with increasing sonication power (Fig. 5).

Control culture and low-intensity sonicated culture were characterized by a roughly constant pellet concentration of around 25 pellets mL^{-1} after the first 100 h (Fig. 5). During high-intensity sonication, the pellet concentration dropped to nil by 140 h. Disintegration of pellets did not lead to fungal death, as biomass continued to grow at a rate that was comparable to control culture (Fig. 2).

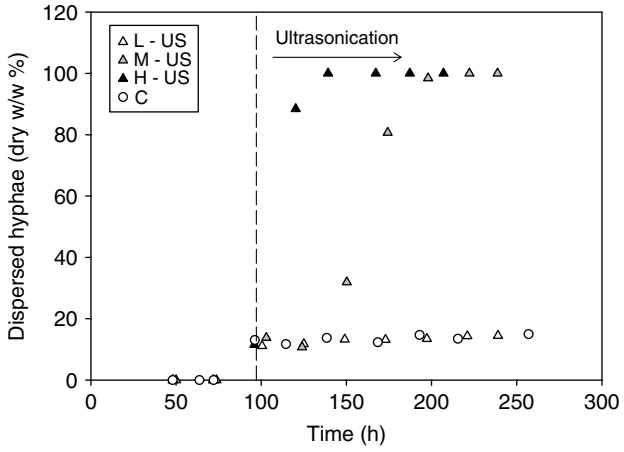

Figure 4. Fraction of dispersed hyphae (dry w/w%) in the broth *versus* fermentation time. L-US: low sonication intensity; M-US: medium sonication intensity; H-US: high sonication intensity; C: control (no sonication).

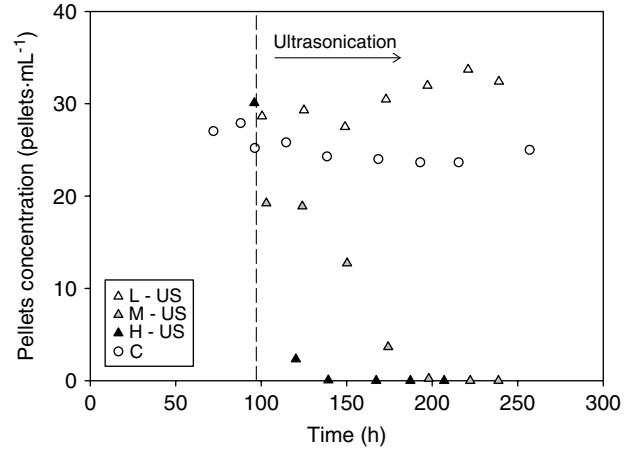

Figure 5. Pellet concentration *versus* fermentation time. L-US: low sonication intensity; M-US: medium sonication intensity; H-US: high sonication intensity; C: control (no sonication).

Effects of intensity of mechanical agitation on pellet morphology of *A. terreus* have been extensively investigated in stirred tank bioreactors.[4,5] Mean pellet diameter d_p (μm) was found to decrease with increase in the total specific power input (P/V), as follows:

$$d_p = 931.8\left(\frac{P}{V}\right)^{-0.42} \quad (1)$$

where P (W) is total power input and V is the volume (m^3) of broth. Equation (1) applied for power input values of up to about 2000 W m^{-3}, but even at this power input the broth contained stable pellets that were roughly 800 μm in diameter. In contrast, during medium- and high-intensity sonication, the power input was ≥2870 W m^{-3}, and hence no pellets survived for long even though the viability of the biomass was not affected. Unlike at the relatively low power levels in stirred tanks where the pellet size reduces due to erosion of external surface,[4,5] sonication at medium and high power intensities produced outright rupture of the pellets.

Lovastatin production

Lovastatin concentration profiles of the various fermentations are shown in Fig. 6. Sonication above the low-intensity level substantially and adversely affected production of lovastatin relative to control fermentation (Fig. 6). As noted previously, biomass concentration and growth were not affected by sonication intensity. It appears, therefore, that ultrasound has the potential to influence production of secondary metabolites without affecting growth metabolism. Earlier work has shown that lovastatin is preferentially produced by *A. terreus* during pelleted growth,[4,5] possibly because of the microenvironment in the pellets. Sonication at medium and high intensities disrupted pellets and this may have indirectly affected the production of lovastatin.

Lovastatin production in high-intensity sonicated culture (H-US, Fig. 6) ceased around 10 h after the initiation of sonication. At medium-intensity sonication (M-US, Fig. 6), lovastatin production ceased around 60 h after sonication commenced (Fig. 6). Although the final lovastatin titer values in high-intensity and medium-intensity sonicated runs were comparable at ~60 mg L^{-1}, production was suppressed sooner in the high-intensity sonicated culture (Fig. 6).

Time at which sonication commences, the duration of sonication, and whether the sound is applied intermittently are known to affect other fermentations.[2] For example, in a simultaneous saccharification and fermentation (SSF) process Wood and co-workers[26] showed that continuous sonication adversely affected

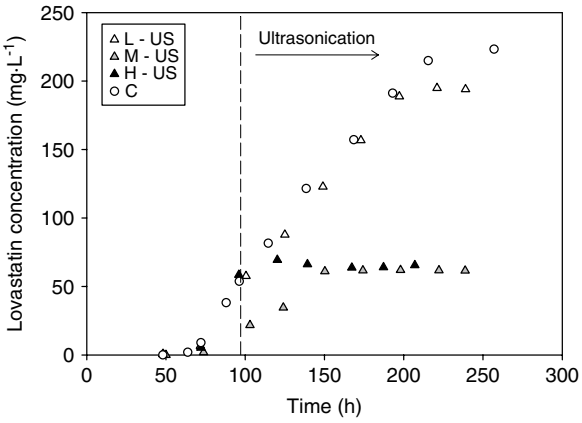

Figure 6. Lovastatin concentration *versus* fermentation time. L-US: low sonication intensity; M-US: medium sonication intensity; H-US: high sonication intensity; C: control (no sonication).

the fermentation, so that ethanol production was not stimulated. Periodic exposure to ultrasound was more beneficial for saccharification than continuous exposure.[26]

Future experiments will explore the use of periodic sonication in attempts to enhance mass transfer of lovastatin from pellets to the surrounding liquid, without damaging the morphology of pellets.

Broth rheology

Rheology of fungal broths is influenced by numerous factors, including the concentration of biomass; relative proportions of the pelleted and free hyphal solids; hyphal strength and branching; growth history; and presence of dissolved polymers. For non-Newtonian fungal broths, rheology is typically characterized by Ostwald–de Waele's power law model. The power law model defines rheological behavior in terms of the flow behavior index n and the consistency index K. The latter is a measure of the 'thickness' of the fluid. The power law model fitted well to the rheological data obtained for the fermentation broths in this work. Figure 7 shows the values of K and n for broth samples taken at various times during fermentations.

In all cases, including the non-sonicated control fermentation, the K-value dropped steadily with increasing fermentation time after the first 100 h (Fig. 7a). During the same period there was relatively little biomass growth (Fig. 2) because of substrate limitation. The fraction of dispersed hyphae did not change much after the first 100 h in the control fermentation and the culture carried out at the low sonication intensity (Fig. 4).

In many fungal fermentations, presence of a high proportion of the biomass as pellets actually reduces the value of K compared with when the biomass is present predominantly as dispersed hyphae. In the present case, in broths that were sonicated at medium and high intensity, the fraction of dispersed hyphae became quite high soon after sonication commenced (Fig. 4), but the K-value of the broth continued to decline (Fig. 7a). The K-values of

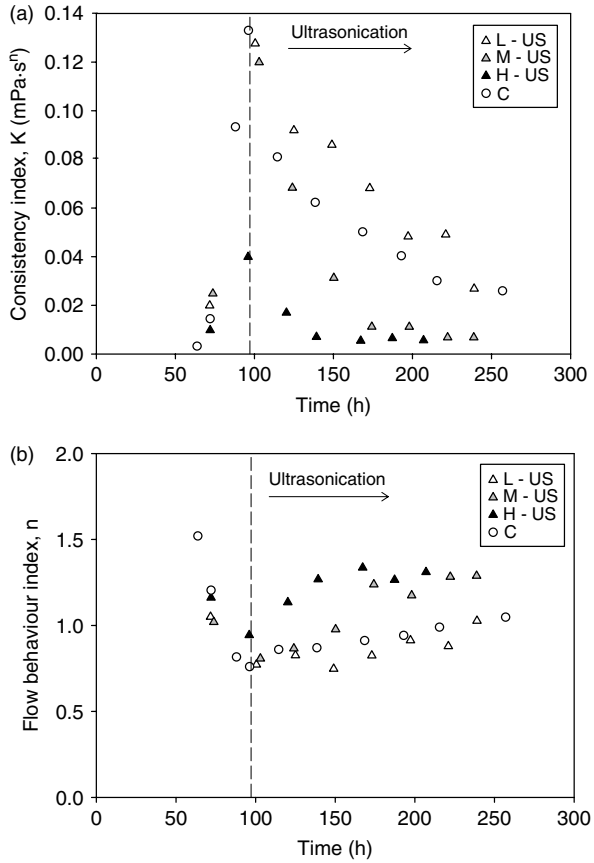

Figure 7. Power law parameters *versus* fermentation time: (a) consistency index, K; (b) flow behavior index, n. L-US: low sonication intensity; M-US: medium sonication intensity; H-US: high sonication intensity; C: control (no sonication).

broths sonicated at medium and high intensity declined faster with fermentation time than did the K-values of control broth and low-intensity sonicated broth (Fig. 7a). This was because pellet rupture due to sonication increased the proportion of small unbranched free hyphae in the culture bulk (Fig. 4) and reduced the concentration of pellets (Fig. 5).

In control culture, although a high proportion of the fungal biomass formed pellets, some hyphae remained in the broth as free filaments or small fluffy clumps. The proportion of pellets to hyphae changed with the progress of the batch fermentation. Around 100 h of cultivation, when the pellet diameter reached its maximum value, the pelleted morphology represented about 85% of the biomass. The 15% of the biomass that was present as free branched hyphae was sufficient to cause extensive interactions among pellets by entangling with the external hyphae in the pellets and thus causing the pellets to bridge. This effect of the free hyphae greatly contributed to making the broth highly viscous. A similar effect occurred in cultures that were not intensely sonicated (Fig. 7a). Later in the fermentation, a limiting amount of nitrogen prevented further growth of hyphae and actually caused some lysis of the free filaments and a reduction in size of the pellets. Around 200 h, pellet–pellet interactions were

reduced sufficiently to greatly reduce the K-value of the broth (Fig. 7a).

In all cases, the n-values of the broths were similar during the first 100 h (no sonication) (Fig. 7b), as expected. The media in the different runs had identical compositions and were identically inoculated. In the first 100 h, the n-values declined to around 0.8; that is, the broths changed from having an initially dilatant rheology to being shear-thinning fluids (Fig. 7b). During this period, the biomass concentration increased (Fig. 2), the average pellet diameter increased (Fig. 3a), and the pellet filament ratio decreased (Fig. 3b).

After 100 h, the n-values increased gradually with fermentation time, but the rate of increase was higher during medium- and high-intensity sonication when compared with the control (Fig. 7b). Soon after the start of sonication and the accompanying loss of pellets, the n-value of the broths of the runs M-US and H-US rose to above unity, indicating a shear-thickening fluid (Fig. 7b). In contrast, in the control culture and L-US broth the n-values were substantially less than unity between 100 h and 200 h of the culture (Fig. 7b), indicating a strongly shear-thinning broth. Clearly, therefore, sonication at medium and high intensities affected broth rheology, but the precise mechanisms behind this remain unclear. A reduction in hyphal length caused by ultrasound could have reduced entanglement of filaments.

CONCLUSIONS

Continuous sonication at power input values of up to 4783 W m^{-3} did not affect the growth rate or biomass yield of *Aspergillus terreus* relative to non-sonicated controls. Sonication at >957 W m^{-3} power input greatly affected fungal morphology. Substantial pelleted growth that was characteristic of control fermentation was entirely replaced by biomass growth as dispersed hyphae. Sonication adversely impacted lovastatin production. At sonication power input value of 4783 W m^{-3} the final lovastatin concentration was about 28% of control fermentation. In view of these results, ultrasound clearly has the potential to alter growth morphology and metabolite production of filamentous fungi. Sonication has the potential to beneficially influence production of fungal metabolites in some cases, particularly those that might require the fungal morphology to be controlled in a freely dispersed form. A sonication power input of ≥ 957 W m^{-3} is required to produce measurable effects on morphology of *A. terreus*.

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