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# Effects of pellet morphology on broth rheology in fermentations of *Aspergillus terreus*

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## Abstract

Effects of pellet morphology on broth rheology are reported for pelleted submerged cultures of the lovastatin producing filamentous fungus *Aspergillus terreus*, growing in fluidized bed and stirred tank bioreactors. The pellet diameter and compactness were affected by the agitation intensity of the broth; however, the total biomass productivity was not affected. In fluidized beds and stirred tanks with agitation intensity of up to 300 rpm (impeller tip speed of 1.02 m s$^{-1}$), the fungal pellets were stable at diameters of up to about 2300 μm. In more intensely agitated stirred tanks (≥600 rpm; impeller tip speed of ≥2.03 m s$^{-1}$), the stable pellet size was only about ≤900 μm. The biomass concentration and the pellet diameter were the main factors that influenced the flow index and the consistency index of the power-law broths. Because the biomass productivity was the same in all experiments in a given type of reactor and the oxygen concentration was kept at ∼400% of air saturation, the pellet size and morphology were not influenced by oxygen mass transfer effects. Pellets were always dense in the core region and no necrosis of the biomass occurred.

*Keywords:* Agitation; *Aspergillus terreus*; Filamentous fungi; Fluidized bed bioreactors; Pellet morphology; Rheology

## 1. Introduction

The metabolic performance of a microbial culture in the bioreactor depends strongly on complex interactions of the various operating conditions. For example, the agitation intensity, the microbial species being cultured, the nutrients type and supply determine the bulk rheology and cellular morphology. Rheology in turn affects supply of nutrients, specially oxygen, and the ease of mixing of the broth. Rheology–morphology relationships are particular relevant in fermentations involving filamentous fungi and bacteria. The specific growth morphology produced 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 [1–3]. Excessive hydrodynamic shear stresses are known to damage mycelial hyphae and pellets [4], but much lower shear stresses are sufficient to influence growth morphology [1,3,4].

The available literature on the effects of mechanical forces on fungal morphology is mostly focused on mycelial cultures consisting of clumps and freely dispersed hyphae [5–9]. In general, little is known about the influence of shear stresses on pelleted fungal cultures [10]. Pelleted growth produces broths that are relatively less viscous and therefore easy to mix and aerate in comparison with filamentous growth [2]. This work is aimed at studying the relationship between the pellet morphology, as influenced by agitation intensity, and the bulk rheology of broths of the filamentous microfungus *Aspergillus terreus*. These effects were examined in a conventional stirred tank fermenter and a fluidized bed bioreactor. In both systems, the dissolved oxygen concentration was held constant at 400% of air saturation, to separate the influence of agitation from the oxygen transfer effects. The stirred tank reactor was operated at 300, 600 and 800 rpm. Fluidized bed reactor was aerated at 1 vvm. The pellet morphology and bulk

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broth rheology were measured for various agitation regimens in the bioreactors.

## 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 fermentation stage, and used for further inoculation of the corresponding bioreactor, either a 5-L working volume stirred tank fermentor or a 17-L fluidized bed reactor. 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 fermentations, seed culture flasks (250 mL) were used to inoculate the bioreactor operated at 28 °C. Fermentations 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. For both culture systems, 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 (STR)

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.

### 2.4. Fluidized bed fermentations (FBR)

Fermentations were carried out in a fluidized bed reactor of 20 L of total volume (17 L of working volume). The aspect ratio was 6. Gas was sparged at 1 vvm using a perforated plate with 150 holes of 1.5 mm diameter. The reactor was fitted with a jacket for temperature control. Fermentations were carried out at 28 °C. The top degasing zone of the fluidized bed column had a jacket of its own and this was cooled at 4 °C to prevent wall growth. The culture medium was the same as used in the stirred tank fermentations, but nitrogen and carbon concentrations were reduced to one-third (1/3) of the standard level. This was done to reduce the maximum attained biomass concentration in the pneumatically mixed bioreactor. Three runs were performed to demonstrate reproducibility.

### 2.5. Rheological measurements

Rheological parameters ($K$, $n$) were measured using a programmable rotational viscometer (Brookfield DV-II+ with standard vane spindles; Brookfield, Middleboro, MA, USA). All measurements were carried out at 28 °C following the method described by Casas López et al. [11]. Rheological tests were performed using the standard vane spindle V-72 (Brookfield, Middleboro, MA, USA), 21.67 mm diameter × 43.33 mm height in a glass vessel of 35 mm diameter, filled to 70 mm. Calibration constants for this vane were determined to be $c = 301.84$ and $k_i = 7.5994$ [11].

### 2.6. Morphological measurements

The fungal pellet morphology was characterized using image analysis [12]. Prior to imaging, each sample of the fermentation broth was processed as follows: 10 mL of sample was decanted and washed twice with 20 mL of distilled water. Within a sample, 100 objects were analyzed for each determination. The image was captured with a CMOS camera (Evolution LC Color; Media Cybernetics, Inc., Silver Spring, MD, USA) mounted on an inverted microscope (Leica DMIL) 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 [10]. 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 [10].

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 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.

### 2.7. Biomass determination

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.

## 3. Results and discussion

After inoculation of the bioreactor with a seed culture of pellets of ~1200 μm mean diameter, the pellet growth and fragmentation patterns observed are shown in Fig. 1 for both the stirred tank (STR) and fluidized bed (FBR). For both reactors, the behavior was essentially identical so long as the agitation speed in the STR did not exceed 300 rpm. Thus, in both cases, the pellets grew in size to attain a steady state diameter of about 2300 μm. In the stirred tank, for agitation intensities of 600 and 800 rpm, the pellet size declined from the instance of inoculation to attain an average steady state diameter of around 900 and 700 μm, respectively. Clearly, in the FBR and the STR agitated at 300 rpm, the turbulence was sufficiently gentle that pellets of ~2300 μm diameter could exist stably.

The known mechanisms of size reduction include surface erosion of the pellet and its outright rupture [10]. How rapidly the pellet size declines to the steady state value depends also on the initial size of the pellet [4]. As the decline in pellet size (Fig. 1) was gradual and progressive, it was likely caused by erosion of the pellet surface rather than outright rupture. The mean pellet diameter in all reactors correlated well with the power input per unit of bulk volume (Fig. 2), confirming that $P_g/V$ influences the dimensions of suspended pellets irrespective of the kind of bioreactor and scale of operation [11]. For both types of reactors, irrespective of the agitation speed and the mode of aeration (air or oxygen enriched air) used, the pellet diameter correlated with the specific power input according to the following equation (Fig. 2):

$$D_p = 931.8\left(\frac{P_g}{V}\right)^{-0.42}$$

where $D_p$ is the average pellet diameter, $P_g$ is the total power input (including that from expansion of the sparged gas) and $V$ is the volume of the broth in the bioreactor.

For all operating regimens and reactors, the filament ratio declined from near unity at inoculation to about 0.4 during the first 72 h (Fig. 3) and stabilized at this value, except in the intensely agitated (800 rpm) stirred tank. Thus, even low-level agitation that did not reduce the initial pellet size, 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. The observed reduction in the filament ratio is likely purely an effect of the fluid mechanical forces. In the

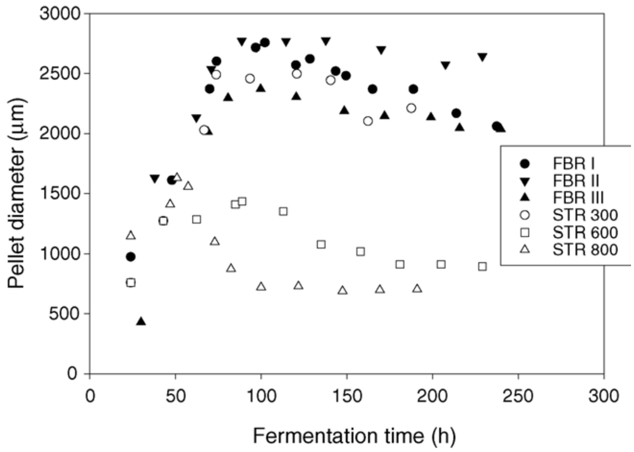

Fig. 1. Pellet diameter versus fermentation time in the fluidized bed (FBR) and the stirred reactors (STR). In separate experiments, the STR agitation speeds were 300 (STR 300), 600 (STR 600) and 800 (STR 800) rpm. The fluid bed reactor operated at an aeration rate of 1 vvm. The FBR I–III are replicates of the same conditions.

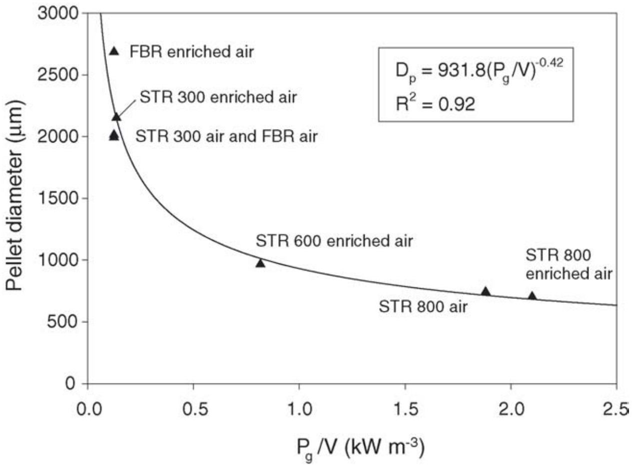

Fig. 2. Pellet diameter vs. total specific power input in various reactors. The three digit number after STR indicates the agitation speed (rpm). The reactors were sparged with air or oxygen enriched air, as indicated.

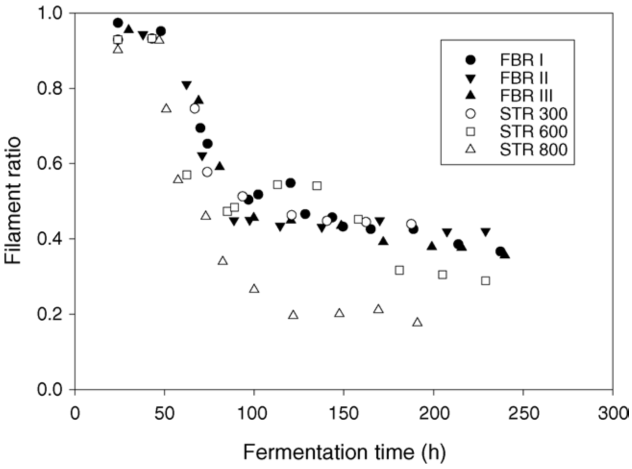

Fig. 3. Filament ratio vs. fermentation time. In separate experiments, the STR agitation speeds were 300 (STR 300), 600 (STR 600) and 800 (STR 800) rpm. The fluid bed reactor operated at an aeration rate of 1 vvm. The FBR I–III are replicates of the same conditions.

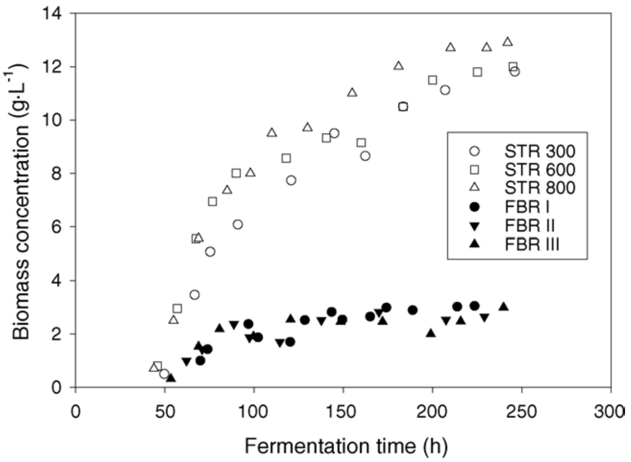

Fig. 5. Biomass concentration vs. time in STR and FBR vessels. The STR agitation speeds were 300 (STR 300), 600 (STR 600) and 800 (STR 800) rpm. The fluid bed reactor operated at an aeration rate of 1 vvm. The FBR I–III are replicates of the same conditions.

intensely agitated STR 800, the decline in the filament ratio was more rapid than in the other reactors and a lower stable value (∼0.2) of the filament ratio was attained. Thus, intense turbulence can rapidly compact fluffy pellets.

Fig. 4 documents the changes in pellet morphology at different stages of culture in the various reactors. At 50 h, that is, soon after inoculation, the pellets from the various reactors were morphologically similar. The pellets were fluffy and the filament ratio was close to 100%. At ≥100 h, the pellets had attained the maximum steady state size in the low agitation environments of the STR (300 rpm) and the FBR. These pellets retained a high level of fluffiness in the peripheral regions. Under the medium intensity agitation of 600 rpm, the pellets at ≥100 h did increase in size a little compared to the situation at 50 h. These pellets were internally compact and the outer fluffy region was quite thin. Under the intense 800 rpm agitation in the STR, the pellets actually decreased in size from about 150 h onwards and the fluffy zones were barely noticeable.

The rheological behavior of culture broth could be described well with Ostwald-deWaelle's power law model. At the early stage of the fermentation, the consistency index ($K$) and the flow index ($n$) values of the broth were essentially the same as for the noninoculated medium. As the fermentation progressed, the biomass concentration increased and this affected the broth rheology. As shown in Fig. 5, within

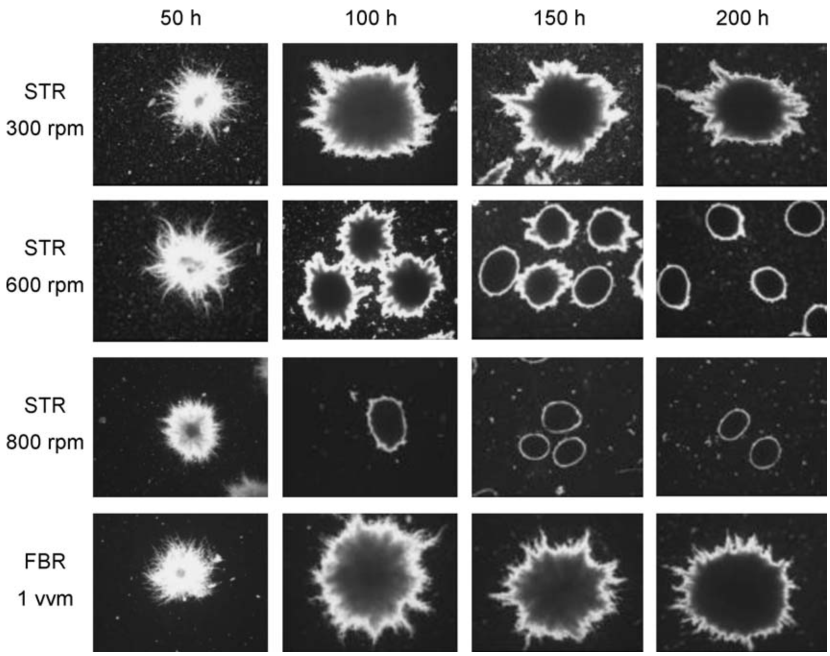

Fig. 4. Pellet morphology at different times in the STR and FBR. The magnification is 40× in all cases.

a given type of reactor (either STR or FBR), the operation conditions did not influence the biomass concentration profiles (Fig. 5). The relatively high biomass concentration in the STRs were because of the richer medium used (see Section 2) in comparison with the medium used in FBRs. Although the biomass profiles within a reactor type were not affected by the agitation regimen (Fig. 5), the different agitation environments produced broths that were rheologically different.

The variation of the flow index ($n$) with fermentation time is shown in Fig. 6a. In all cases involving the FBR, the $n$-value initially declined as the pellet size increased. Once the pellets had attained a stable size, a corresponding stable value of flow index was obtained (Fig. 6a).

At 300 rpm agitation, the pellet morphology and size in the STR were virtually the same as in the FBR (Figs. 1, 3 and 4), but the $n$-values of the broth were dramatically different (Fig. 6a) simply because of a much higher biomass concentration in the STR (Fig. 5) and a consequent higher concentration of pellets. In the STR, the small compact pellets produced under intense agitation (800 rpm, Fig. 6a) produced shear-thickening broths ($n > 1$; Fig. 6a) compared to the shear-thinning broths ($n < 1$) obtained for 300 rpm agitation.

Irrespective of the reactor or agitation regimen, the $K$-value initially increased with the progress of the fermentation, attained a peak and subsequently declined (Fig. 6b). This happened even though the biomass concentration continuously increased during the various fermentations (Fig. 5). Clearly, the pellet size and concentration effects were influencing the value of $K$.

An analysis of variance was carried out separately on data from STR and FBR (Table 1) to clearly identify the variables that were influencing the broth rheology. Data included in this analysis comprised $K$ and $n$-values of broths at different biomass concentrations, mean pellet diameters and mean filament ratios. For the consistency index $K$, the mean pellet diameter and mean filament ratio had a significant effect at 95% confidence (i.e. $P$-values of less than 0.05) in both the STR and FBR (Table 1); however, in the STR cultures the contribution of pellet diameter to the observed changes in $K$ was greater than the contribution of the filament ratio, as indicated by the $F$-ratio statistics (Table 1). For the flow

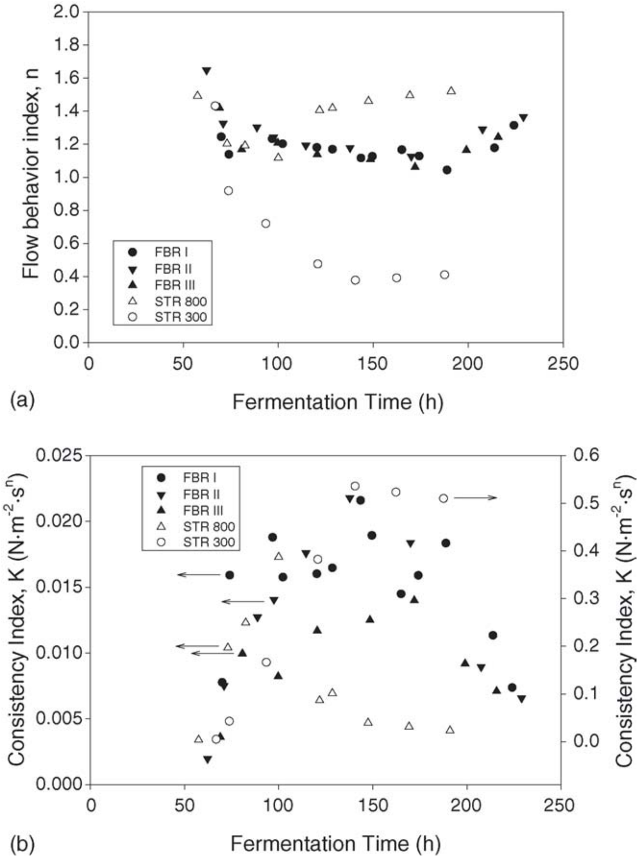

Fig. 6. The $n$-values (a) and $K$-values (b) of the broths as a function of the fermentation time in various reactors. The STR 300 and STR 800 were agitated at 300 and 800 rpm, respectively. The FBR I–III are replicates of the same conditions.

Table 1
Analysis of variance for power law parameters measured in STR and FBR

| Source | Stirred tank experiments | | | | | Fluidized bed experiments | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Sum of squares | Df | Mean square | $F$-ratio | $P$-value | Sum of squares | Df | Mean square | $F$-ratio | $P$-value |
| Consistency index, $K$ | | | | | | | | | | |
| Biomass concentration | 0.0515 | 2 | 0.0257 | 2.14 | 0.1419 | 0.0001 | 2 | 0.0000 | 1.96 | 0.1476 |
| Mean pellet diameter | 0.2425 | 2 | 0.1212 | 10.07 | 0.0008 | 0.0003 | 2 | 0.0001 | 5.59 | 0.0052 |
| Mean filament ratio | 0.1117 | 2 | 0.0558 | 4.64 | 0.0209 | 0.0002 | 2 | 0.0001 | 4.34 | 0.0159 |
| Residual | 0.2649 | 22 | 0.0120 | | | 0.0022 | 86 | 0.0000 | | |
| Total (corrected) | 0.8407 | 28 | | | | 0.0032 | 92 | | | |
| Flow behavior index, $n$ | | | | | | | | | | |
| Biomass concentration | 0.0003 | 2 | 0.0002 | 0.00 | 0.9980 | 0.0663 | 2 | 0.0331 | 2.16 | 0.1211 |
| Mean pellet diameter | 1.2284 | 2 | 0.6142 | 8.09 | 0.0023 | 0.2181 | 2 | 0.1090 | 7.12 | 0.0014 |
| Mean filament ratio | 0.9901 | 2 | 0.4951 | 6.52 | 0.0060 | 0.0434 | 2 | 0.0217 | 2.16 | 0.2483 |
| Residual | 1.6709 | 22 | 0.0759 | | | 1.3176 | 86 | 0.0153 | | |
| Total (corrected) | 5.8370 | 28 | | | | 1.7981 | 92 | | | |

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

Table 2
Analysis of variance for power law parameters

| Source | Consistency index, *K* | | | | | Flow behavior index, *n* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Sum of squares | Df | Mean square | *F*-ratio | *P*-value | Sum of squares | Df | Mean square | *F*-ratio | *P*-value |
| Biomass concentration | 0.7275 | 4 | 0.1819 | 72.82 | 0.0000 | 4.6429 | 4 | 1.1607 | 41.27 | 0.0000 |
| Mean pellet diameter | 0.3425 | 4 | 0.0856 | 34.29 | 0.0000 | 2.7413 | 4 | 0.6853 | 24.37 | 0.0000 |
| Mean filament ratio | 0.0094 | 4 | 0.0024 | 0.94 | 0.4424 | 0.1791 | 4 | 0.0448 | 1.59 | 0.1817 |
| Residual | 0.2722 | 109 | 0.0025 | | | 3.0658 | 109 | 0.0281 | | |
| Total (corrected) | 1.1577 | 121 | | | | 10.3318 | 121 | | | |

Data include STR and FBR fermentations. All *F*-ratios are based on the residual mean square error.

behavior index *n*, only the pellet diameter had a significant effect on broths produced in the FBR.

When the data from the STR and FBR were pooled and an analysis of variance performed, the biomass concentration and the pellet diameter were seen to affect the *K* and *n* values (i.e. *P*-value of <0.05 in Table 2). The filament ratio no longer effected the rheological parameters (*P*-value of >0.05 at the 95% confidence level).

## 4. Conclusions

The following specific conclusions can be drawn for pelleted submerged fermentations of *A. terreus*:

(1) In fluidized beds and stirred tanks agitated at up to 300 rpm (impeller tip speed up to $1.02\,\mathrm{m\,s^{-1}}$), the hydrodynamic shear regimens are comparable and permit stable existence of pellets of up to $\sim$2300 μm in diameter.
(2) Intense shear forces in stirred tanks ($\geq$600 rpm; impeller tip speed of $\geq 2.03\,\mathrm{m\,s^{-1}}$) fragment pellets of $\sim$1200 μm initial size to a stable size of $\leq$900 μm.
(3) The compactness or fluffiness of pellets are affected by the agitation intensity. Small, dense pellets form under intense agitation ($\geq$600 rpm).
(4) The broth rheology parameters *K* and *n* are influenced by the concentration of the biomass, the average pellet size and, to a lesser extent, the fluffiness of the pellet.

All these effects are completely independent of any oxygen transfer related effects, as within a given reactor the biomass concentration is not affected by the agitation intensity over the range investigated.

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