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Study of the Rheological Properties of a Fermentation Broth of the Fungus *Beauveria bassiana* in a Bioreactor Under Different Hydrodynamic Conditions

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Fermentation with filamentous fungi in a bioreactor is a complex dynamic process that is affected by flow conditions and the evolution of the rheological properties of the medium. These properties are mainly affected by the biomass concentration and the morphology of the fungus. In this work, the rheological properties of a fermentation with the fungus *Beauveria bassiana* under different hydrodynamic conditions were studied and the rheological behavior of this broth was simulated through a mixture of carboxymethyl cellulose sodium and cellulose fibers (CMCNa-SF). The bioreactor was a 10 L CSTR tank operated at different stir velocities. Rheological results were similar at 100 and 300 rpm for both systems. However, there was a significant increase in the viscosity accompanied by a change in the consistence index, calculated according to the power law model, for both systems at 800 rpm. The systems exhibited shear-thinning behavior at all stir velocities, which was determined with the power law model. The mixing time was observed to increase as the cellulose content in the system increased and, consequently, the efficiency of mixing diminished. These results are thought to be due to the rheological and morphological similarities of the two fungal systems. These results will help in the optimization of scale-up production of these fungi.

Keywords: Rheology, hydrodynamics, filamentous fungus, simulation bioprocess

The fermentation broth of microorganisms, especially filamentous fungi, is a complex rheological system where the accumulation of biomass or biosynthesized product leads to the continuous modification of the rheological properties of the medium produced in a bioreactor. Moreover, this medium is heterogeneous owing to cavern formation and recirculation. Under these conditions, one of the most important problems to be solved is to establish an adequate flow regime and processing parameters to carry out an optimal fermentation. Another problem arises when microorganisms such as filamentous fungi produced in the fermentation are sensitive to high shear stress [5]. The thread-like form of these fungi creates tridimensional network systems that produce highly viscous and non-Newtonian fermentation soups where mass transfer and homogenization are restrained. The implementation of high stir velocities is a common approach to overcoming these problems. However, high mechanical stress can result in cellular damage, leading to fungal cell differentiation [16, 17]. In recent years, there has been an increasing interest in the study of the optimization of the CSTR process conditions in terms of mixing times [5, 8, 14, 15, 18], power consumption [4], rheological behavior [1, 6], and chemical and morphological analyses [2, 9, 15]. On the other hand, given the complex morphology of the fungi involved, it is clear that monitoring is required at the macro and microscopic levels (evolution of the flow properties and image analysis) of the broth for proper control of the filamentous solution fermentation process. The optimization of this process is necessary since these types of fungi are of great importance owing to their insecticidal activity, which controls a variety of insect pests. The disadvantage of working with these microbial systems is a high risk of contamination, excessive costs, time constraints, and also the fact that the biomass concentration and morphology of these broths contribute significantly to modification of the rheological behavior of these systems; high viscosity and

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even changes in the pseudoplasticity index (n < 1) [10, 13] limit both the operation and optimization of such systems.

Previous studies have used different concentrations of arboximethyl cellulose (CMC) to try to simulate the rheological behavior of microorganisms [7, 11, 13]. However, this similarity is only attained in the rheological properties, leaving aside morphological considerations. On the other hand, Benchapattarapong *et al*. [2] simulated a *Tolypocladium inflatum* fermentation with Solka-Floc (SF) cellulose fiber and CMC, showing similar morphological and rheological characteristics. These studies reveal that there is a need for more research with this type of microbial system in order to compare SF with other organisms having similar mycelial growth morphologies.

In this study, different concentrations of SF were used to simulate the solid phase (fungal filaments) in combination with sodium carboxymethyl cellulose (CMCNa) in order to attain a viscosity and microstructure similar to that of the fermentation broth of the fungus *Beauveria bassiana*. The main objective of this work was to determine the best hydrodynamic conditions (Reynolds number, Re) with a combination of Maxflo−Rushton impellers to pre-establish geometric conditions in an actual fermentation operation with *B. bassiana*, as well as with CMCNa-SF, to compare the evolution of rheological and morphological properties and to infer mixing times in fermentation broths under different flow conditions.

Materials and Methods

Microorganism and Cultivation Medium

The microorganism used in this study was the filamentous fungus *B. bassiana* (strain MH-04) isolated from maize plants. It was obtained from the Interdisciplinary Research Center for Regional Integral Development-National Polytechnic Institute (CIIDIR-IPN) campus in Durango. The medium used for cultivation had the following formulation (per liter): 40 g glucose, 10 g peptone, 6.8 g KH_2PO_4, 2.5 g $MgSO_4{\cdot}7H_2O$, 0.1 g $CaCl_2$, and 0.02 g $FeCl_3$. The initial pH was adjusted to 5.4 with 2 N NaOH. Fermentation was carried out at stirring speeds of 100, 300, and 800 rpm, with aeration at a rate of 1 vvm, a constant temperature of 30°C, and constant pH of ~5.4 during the entire fermentation. At 12 h intervals, samples were taken from the fermentation broth to determine the concentration of biomass on a dry weight basis, and to follow the evolution of mechanical properties and morphological flow during 5 days of continuous fermentation.

Medium Used for Simulation of Fermentation

Various simulations were performed with SF (Solka-Floc 40 powdered cellulose; International Fiber Corporation) and CMCNa. A mixture of the two, CMCNa-SF, was used to simulate the fermentation of a filamentous fungal solution at low and high concentrations of biomass. Various concentrations of CMCNa-SF were used to assess whether the simulation has the potential to simulate both the rheological properties and morphology of the fungus.

Determination of Mixing Time

Mixing time values were estimated by changes in pH at different concentrations of the CMCNa-SF system under different flow hydrodynamic conditions, using a solution of NaOH as a tracer. The time required for the pH to reach a constant value was monitored, reaching a considerable value of mixing intensity [5]. The latter was determined under the criteria of homogeneity:

$$I = \frac{pH_\infty - 0.5\Delta pH}{pH_\infty} \times 100 = 99\% \tag{1}$$

where $\Delta pH = 0.02$.

In this work, the tracer volume was 1 ml, injected 10 mm from the surface of the liquid (fixed level). Variations in the pH in the bioreactor were recorded by a computer system.

Experimental Equipment

A 10 L (6 L volume) laboratory-scale CSTR-type bioreactor (Biostat model; B. Braun, USA) was used in this study and is illustrated in Fig. 1. The bioreactor mixing system consisted of two baffles and two mixers: a Maxflo type one placed in the top and a Rushton type one at the bottom of the arrow. A fixed distance (C) between the upper and lower agitators of 68 corresponds to the impeller diameter (D), and also to the distance from the bottom of the tank to the first impeller. The liquid height (H) was 0.20 m and the tank diameter (T) was 0.19 m.

Rheological Characterization

Rheological characterization consisted of steady simple shear flow tests applied to the *B. bassiana* fermentation broth and to the model fluids. Flow curves were obtained at different CMCNa-SF concentrations. A controlled stress rheometer (Model AR-G2; TA Instruments) was used with a concentric cylinder geometry (outer cylinder diameter = 21.96 mm, inner cylinder diameter = 20.38 mm, H = 59.50, gap = 500 μm), maintaining a constant temperature of 25°C with the help of a circulatory water bath (Polystat; Cole Parmer).

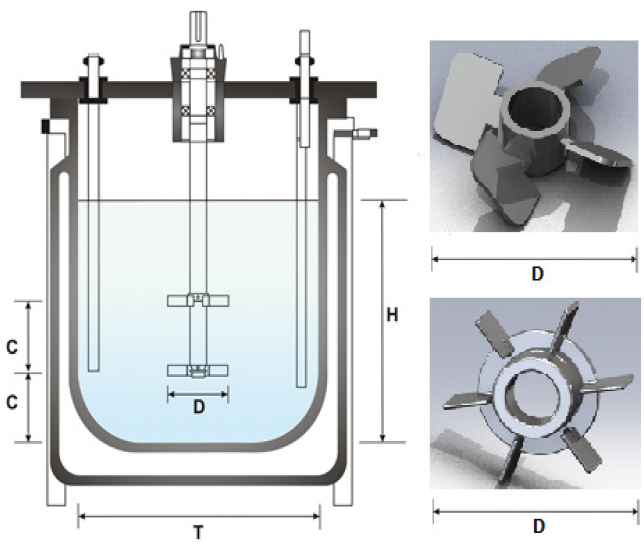

Fig. 1. Specifications of the bioreactor.
Stirred tank type, Maxflo−Rushton impellers.

Rheological parameters in simple shear flow were monitored at a shear rate ranging from 0.1 to 50 s^{-1}. Experimental data for each curve was adjusted by the power law model [3, 12], given by Eq. (2):

$$\eta = K\dot{\gamma}^{n} \quad (2)$$

where K is the consistency index (Pa s^n) and n is the shear thinning index (-).

All rheological measurements were carried out in duplicate. The experimental rheological data were obtained directly from the TA Rheology Advantage Data Analysis software V.5.7.0 (TA Instrument Ltd., Crawley, UK).

Morphological Characterization

Sample morphology was evaluated to observe conformational changes in the fermentation broth. In this technique, the sample is illuminated with short wavelength light, some of which is absorbed by the sample. An electron microscope lamp with a built-in field diaphragm with 30 W reflected light, a wavelength of 390–420 nm, and a 40× objective were used. This equipment provided only black and white images. The analysis was performed at least in triplicate.

RESULTS AND DISCUSSION

Growth Kinetics

Fig. 2 shows the evolution of the increase in biomass concentration during fermentation with *B. bassiana.* At a stirring speed of 100 rpm, a maximum of 6.3 g/l of biomass on a dry basis was obtained at 84 h of fermentation, and the system was observed to sporulate at 120 h. Surprisingly, the maximum yield of biomass appeared when the dissolved oxygen value reached a steady minimum value. In the case of the fermentation at 300 rpm, the biomass concentration reached a maximum of 20 g/l on a dry basis at 108 h, a value that is more than 2-fold that for fermentation at 100 rpm. Finally, fermentation at 800 rpm generated a maximal biomass production of 25 g/l (the highest yield of all three experiments) on a dry basis at 84 h. The drastic decrease in dissolved oxygen was mainly due to the fact that viscosity increases with increasing biomass in the fermenter, significantly reducing the availability of oxygen in the fermentation broth.

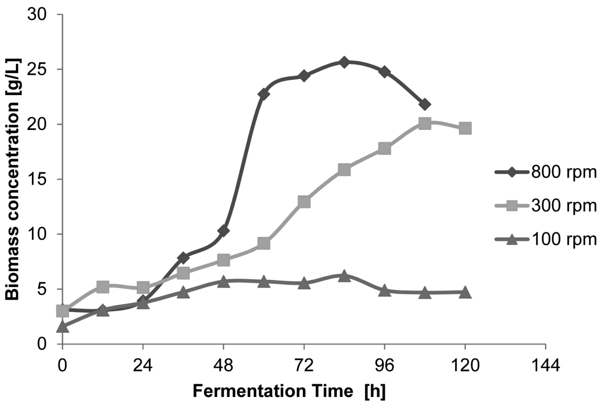

Fig. 2. Increase in biomass concentration during fermentation with *B. bassiana* at 100, 300, and 800 rpm.

Rheological Properties of Fermentation Broth for Fermentation by *B. bassiana* and Simulation with CMCNa-SF

Fermentation is largely affected by the rheological properties of the medium. These properties are primarily controlled by the biomass concentration and the morphology of the fungus. The accumulation of biomass leads to an increase in viscosity of the fermentation broth. Not only does the concentration increase but the morphology of the fungi evolves and becomes more elongated (filamentous). These filamentous fungi tend to orient in the direction of the flow and to structure themselves, forming random networks, thus exhibiting a non-Newtonian behavior of the pseudoplastic type ($n < 1$) as shown in Fig. 3A. Phenomena such as yield stress may also appear. For the fermentation at 100 rpm, there was an increase in the apparent viscosity of the medium as the biomass concentration increased, with consistence indexes on the order of 0.017 to 0.729 Pa s^n with Re $\leq$ 160 (transition flow regime) according to the power law model. Similar conditions were used for the CMCNa-SF system; the results are shown in Fig. 3B, with apparent viscosities ranging from 0.015 to 0.605 Pa s^n at a rate of shear thinning flow ($n < 1$) similar to that observed

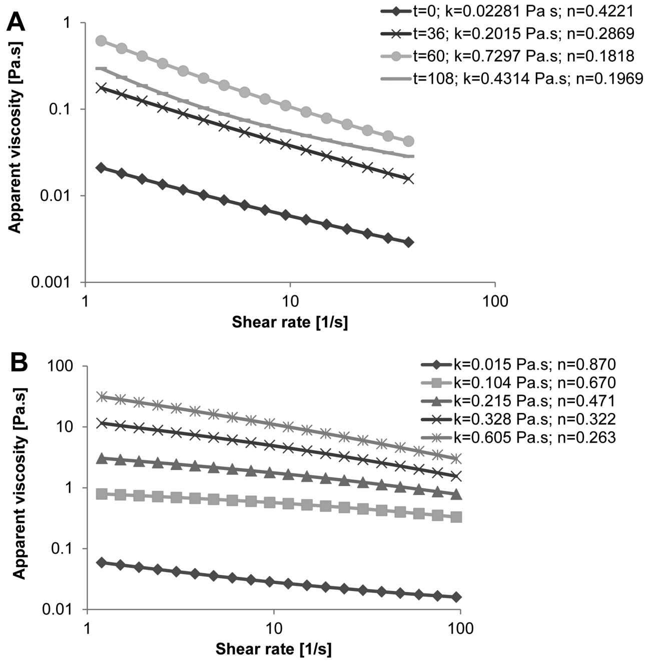

Fig. 3. (**A**) Rheological behavior during fermentation with *B. bassiana* at 100 rpm, and (**B**) simulation of rheological behavior of fermentation at 100 rpm with CMCNa-SF, both adjusted by the power law model.

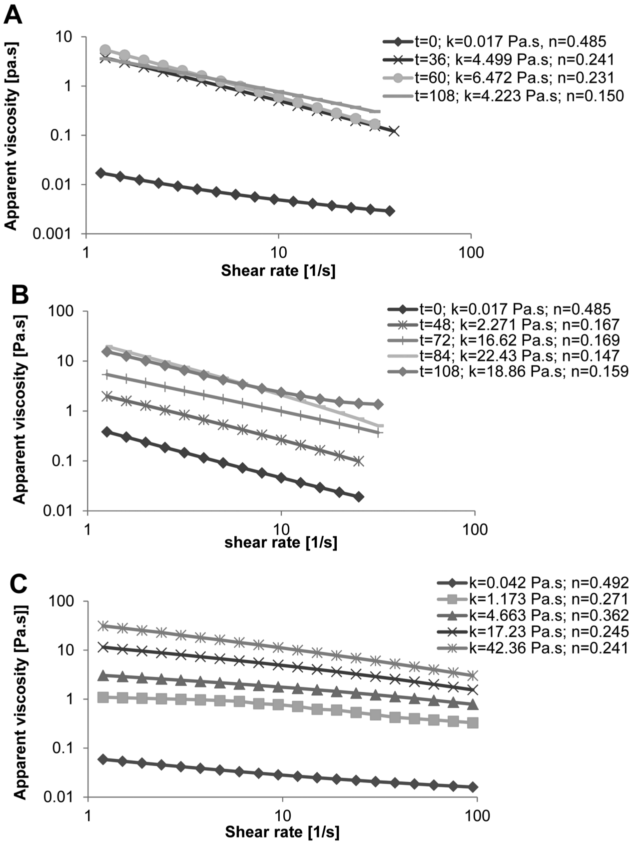

Fig. 4. (**A**) Rheological behavior during fermentation with *B. bassiana* at 300 rpm and (**B**) 800 rpm, and (**C**) rheological behavior of the simulation with CMCNa-SF, fitting the power law model.

for the fungus. It is important to mention that in the case of the CMCNa-SF system, only the concentration was changed to obtain a rheological behavior similar to that of *B. bassiana*.

For the fermentation with *B. bassiana* at 300 rpm (Fig. 4A), an increase in biomass concentration was obtained compared with that at 100 rpm, with apparent viscosities ranging from 0.017 to 6.472 Pa s^n at different fermentation times. The flow regime was found to be in the transition zone (Re ≤ 122). The system under those conditions was found to correspond to CMCNa-SF with concentrations ranging between 3(%) CMCNa + 15 (g/l) SF with an apparent viscosity of 4.663 Pa s^n, and 4(%) CMCNa + 20 (g/l) SF with an apparent viscosity of 17.230 Pa s^n, as shown in Fig. 4B. Table 1 shows the rheological parameters for the different concentrations used in the CMCNa-SF simulation.

The maximal biomass yield and also apparent viscosity were found for the fermentation at 800 rpm. Flow indexes ranging from 0.017 to 22.430 Pa s^n according to the power law model were obtained, which corresponds to a transition flow regime (Re ≤ 245) (Fig. 4B). This behavior was simulated with CMCNa-SF at concentrations between 4(%) CMCNa +20 (g/l) SF and 5(%) CMCNa +25 (g/l) SF, with apparent viscosities between 17.230 and 42.360 Pa s^n, respectively (Fig. 4C and Table 1).

These results are of great importance because of the possibility of simulating the complex viscous behavior of the fermentation broth for this type of fungus with a system with similar morphology by only modifying the concentration. In this way, optimization, control, and predictions under different hydrodynamic conditions are easier; fermentation times are long and monitoring is tedious, whereas the model system enables the prediction of rheological behavior without having to run a fermentation experiment. Unlike the study reported by Benchapattarapong *et al*. [2], where the concentration of the liquid phase was maintained constant and the solid concentration was varied accordingly as the amount of biomass increased in the broth, here, not only the solid phase (SF) but also the liquid phase (CMCNa) were changed to model the rheological behavior of the fermentation broth. Consequently, not all organisms have the same rheological behavior, as can be seen in the fermentations at 100, 300, and 800 rpm (Fig. 3 and 4), with their respective simulations with CMCNa-SF. According to the data obtained for the fermentation broths, it is possible to simulate the flow behavior of these complex systems with a model system of similar morphology, and the viscosity curve is a function of concentration of both CMCNa and SF in the transition flow regime.

Morphologically, it was found that the structure of SF particles is similar to that of *B. bassiana*. This similarity can be seen in Fig. 5, in which micrographs of the fungus at 72 h of fermentation and simulation with CMCNa-SF at a concentration of 3(%) CMCNa + 15 (g/l) SF are compared.

Table 1. Different concentrations of CMCNa-SF for the simulation of *B. bassiana* fermentation broth.

(a)	0.1(%)CMC+5(g/l)SF	0.2(%)CMC+10(g/l)SF	0.3(%)CMC+15(g/l)	0.4(%)CMC+20(g/l)SF	0.5(%)CMC+25(g/l)SF
K	0.015	0.104	0.215	0.328	0.605
n	0.870	0.670	0.471	0.322	0.263
(b)	1(%)CMC+5(g/l)SF	2(%)CMC+10(g/l)SF	3(%)CMC+15(g/l)	4(%)CMC+20(g/l)SF	5(%)CMC+25(g/l)SF
K	0.042	1.173	4.663	17.230	42.360
n	0.492	0.271	0.362	0.245	0.241

(a) Low viscosity and (b) high viscosity.

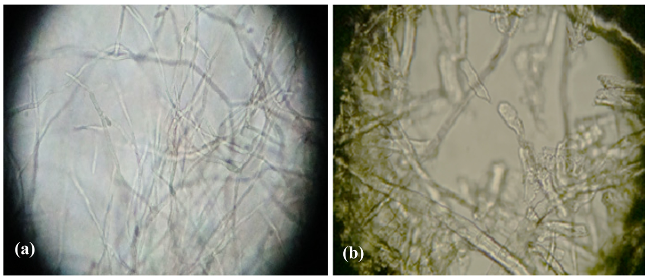

Fig. 5. Micrographs of (a) *B. bassiana* after 72 h of fermentation at 300 rpm, and (b) simulation with CMCNa-SF.

According to this comparison, the model system was found to be appropriate for simulation of the viscous behavior of a real filamentous fermentation.

In the fermentation broths, particle aggregates were observed at 100 rpm, whereas at 300 rpm their presence was minimal, a result that is consistent with the rheological characterization at short times or high flow rates. Finally, aggregate formation was not observed with agitation at 800 rpm owing to a higher biomass concentration and the higher viscosity of the broths. Thus, increasing the stirring speed does not necessarily produce a more uniform system. This was the case for the fermentation at 800 rpm, where a more dominant fungal micelle form was observed; a more heterogeneous system with more viscous fermentation broths is produced, which enhances flow instabilities due to the formation of unmixed zones that are more difficult to control, resulting in a more expensive operation. As reported in the literature [16, 17, 19], the more convenient micellar morphology from an operational standpoint is that of segregated broths, which exhibit predominantly Newtonian rheological characteristics ($n = 1$) and low viscosity, and in principle they are more stable to flow. However, as the size of the segregates increases, the dysfunctional limitations of nutrients delivered to the heart of the segregates (diffusion) cause a decrease in the productivity of the fungus.

The results presented in this work indicate that for the production of filamentous fungi, appropriate agitation speeds (Re) should be considered. Herein, 300 rpm was found to be optimal (transition flow regime $Re \leq 100$); this speed was not too low to cause difficulties in oxygenation, but at the same time, it was not too high to produce highly viscous and therefore heterogeneous systems. Avoiding high speeds of agitation that in an extreme case may even produce yield stress fluids is desirable, for this may result in considerably increased mixing times. In addition, the use of high agitation speeds is expensive and the applied mechanical stress can cause cell damage in the long term and thin the structure of the hyphae, producing cell differentiation [16, 17].

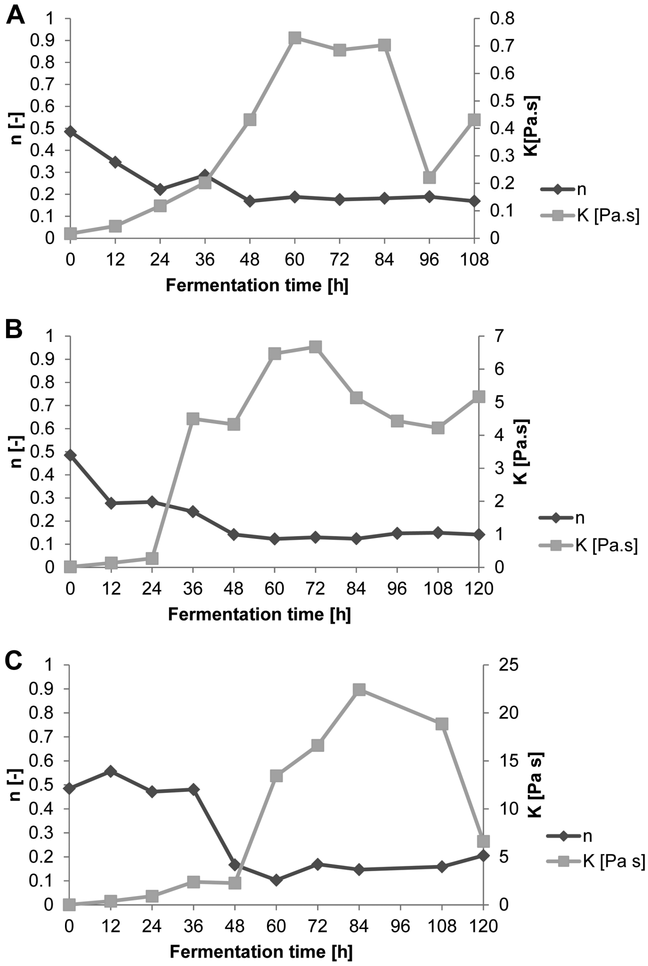

Fig. 6. Rheological behavior depending on the consistency index (K) and flow behavior index (n) of the *B. bassiana* fermentation broth.
(**A**) 100 rpm, (**B**) 300 rpm, and (**C**) 800 rpm.

In Fig. 6, the rheological properties of the *B. bassiana* fermentation broth, that is, the consistency index (K) and flow behavior index (n), are shown as a function of time for different stirring speeds. The flow behavior index n decreased as the amount of biomass increased and, consequently, K increased. In the case of the fermentation at 100 rpm (Fig. 6A), a value of $n = 0.182$ (-), with $K = 0.703$ Pa s^n was obtained at 84 h of fermentation, which corresponds to the maximum biomass concentration. The fermentation at 300 rpm (Fig. 6B) showed a value of $n = 0.130$ (-) with $K = 6.677$ Pa s^n at 72 h with higher biomass production as compared with the fermentation at 100 rpm. Finally, for 800 rpm (Fig. 6C), values of $n = 0.147$ (-) and $K = 22.430$ Pa s^n were obtained at 84 h with the highest production of biomass. These results indicate that the fermentation of *B. bassiana* at the three agitation speeds exhibited an index of non-Newtonian

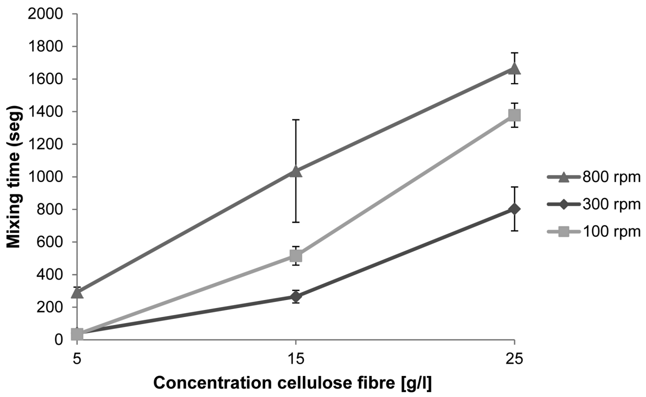

Fig. 7. Mixing time for stirring speeds of 100 and 300 rpm, obtained with CMCNa-SF simulation.

shear thinning flow behavior ($n < 1$), with significant changes in the level of pseudoplasticity, which was calculated according to the power law model. It was also observed that as the stirring speed was increased, both the viscosity and the pseudoplasticity of the fermentation broth increased. This result was expected, especially at 800 rpm, where the morphological evaluation of the fungus revealed a structure in the form of filaments.

This filamentous form (Fig. 6C) often produces three-dimensional networks, causing the formation of highly viscous non-Newtonian broths, where mass transfer and homogenization of the medium are severely limited and therefore of very little practical value.

Simulations of *B. bassiana* Fermentation to Determine Mixing Times.

In Fig. 7, the simulation of *B. bassiana* fermentation using SF cellulose fiber is shown. The mixing time at 100 rpm was around an order of magnitude longer than that at 300 rpm. In both cases, it was observed that with lower concentrations of cellulose fiber [5% (w/v)], the mixing times were reduced. On the other hand, with 25% w/v cellulose fiber (higher apparent viscosity), the mixing time was longer owing to the greater amount of suspended solids that must be promoted or moved in order to homogenize the system; that is, upon increasing the concentration of SF, the mixing time is increased, significantly reducing the efficiency of mixing. According to these results, the best stirring speed is 300 rpm, which resulted in shorter mixing times due to enhancement of the mechanical stability to flow in the medium evidencing a more homogeneous system, whereas at 100 rpm, the system was unstable owing to the sedimentation of suspended solids from SF (low dispersion in the medium). Moreover, the simulation performed at 800 rpm caused instability, indicating the high viscosity of the medium and perhaps yield stress phenomena, and consequently longer mixing times. It should be noted that the mixing time is related to power consumption, and therefore to the mixing energy, which is of vital importance in the design and optimization of such complex rheology systems.

In conclusion, the rheological behavior of the fungus *B. bassiana* was evaluated. The system is a non-Newtonian, shear thinning ($n < 1$) fluid showing significant changes in the level of pseudoplasticity depending on the mixing conditions. The flow behavior of the fermentation broth was shown to be very sensitive to the agitation speed. Flow conditions were affected by the amount of biomass, rheological properties of the broth, the morphology of the fungi and the system homogeneity. The mixing time was highly influenced by the amount of biomass. As biomass production increased, the mixing time increased and thus the efficiency of mixing was significantly reduced. These observations were verified through the CMCNa-SF simulation, which showed very similar flow behavior to that of a real filamentous fungi fermentation. A CMCNa-SF system was used that presented similar rheology and morphology to the fungus under study, and it is a practical system to model the hydrodynamic evolution and characterization of such systems in stirred tanks.

List of Symbols

Re	Reynolds number (dimensionless)
D	diameter of the impeller (m)
pH_∞	pH value corresponding to perfect mixing
ΔpH	pH limits accepted for mixing time determination
I	criterion of homogeneity (%)
t	time (s)
η	viscosity (Pa.s)
K	consistency index (Pa s^n)
$\dot{\gamma}$	shear rate (s^{-1})
n	flow behavior index (-)

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