Mitigation of hydrodynamics related stress in bioreactors: a key for the scale-up of enzyme production by filamentous fungi

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Tamiris ROQUE[1], Frédéric AUGIER[1], Nicolas HARDY[1], Fadhel BEN CHAABANE[1], Alvin NIENOW[2] and Catherine BEAL[3]

[1]IFP Energies nouvelles, Rond-point de l'échangeur de Solaize, BP3, 69360 Solaize, France
[2]School of Chemical Engineering, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom
[3]UMR 782 AgroParisTech INRA, 1 avenue Lucien Brétignières, 78850 Thiverval-Grignon, France

Abstract: Different impellers and stirring conditions are compared at the bench scale in order to identify the relevant hydrodynamic stress parameter that correlates best with the process characteristics, morphology, broth rheology and growth rate as measured during the culture of the filamentous fungi, *T. reesei*, used for the production of cellulases. A modified Energy Dissipation Circulation Function (EDCF) was the most relevant stress parameter, and its use is validated to predict fairly accurately the same process characteristics in an industrial bioreactor, as well as enzyme production. The results are of great importance for the scale-up of cellulase production for bioethanol manufacture from lignocellulosic biomass.

Keywords: Agitation, Fluid dynamic stress, Scale-up, Filamentous fungi, Enzymes.

Introduction

Trichoderma reesei is a filamentous fungi often employed for the production of cellulases to degrade lignocellulosic biomass for second generation bioethanol production. *T. reesei* culture requires high oxygen uptake rates, thus high rates of mass transfer and at the same time, its filamentous morphology induces a high viscosity broth of complex rheology. In addition, this morphology changes during the fermentation process as a consequence of the development of mycelial structures and the increase of the cellular concentration. To insure an appropriate level of dissolved oxygen, the intensity of the stirring may need to be increased to several kW/m^3 of broth if necessary. In this case, in addition to economic issues, the question of damage caused to microorganisms has to be addressed. This question is quite easy to investigate at laboratory scale, but is much more difficult at an industrial one, as the scale-up parameters concerning fluid dynamics stress are still not well known. The enzyme production follows a two-steps process. First the growth of fungi is operated with an excess of sugar to reach a targeted biomass concentration. The end of this step is critical having both the highest oxygen demand and viscosity and thus the greatest specific power input. The second step is operated under sugar limitation with a lower oxygen demand and much lower viscosity due to the metabolism and morphological changes of the fungi [1]. In this context, the objectives of the present study are to:

- Study fluid dynamic stress damage to *T. reesei* during the growth and production steps
- Identify relevant scale-up criteria concerning such damage to the microorganism
- Use the relevant criteria to guide the choice of the mixing parameters at industrial scale.

The study is essentially based on lab-scale experiments operated under various mixing conditions during growth and production steps. Morphology, rheology and biological yields are compared versus different scale-up parameters. Relevant criteria are validated on industrial data acquired on a bioreactor of hundreds of cubic meters.

The presented work is based on the recently published study of Hardy et al. (2017) [2], concerning results obtained during the growth step, and novel unpublished work concerning the production step. The characterization of the impact of the agitation on the enzyme production uses 2D electrophoresis [3] to identify proteome and secretome modifications due to fluid dynamics stress.

Material and methods

Study of the growth step

The growth step was done in batch mode in a 3.5 L bioreactor (2.5 L of culture medium), shown in figure 1.. The conditions of culture and preculture were as described in [1] with the

temperature at 27°C and pH controlled at 4.8 for all the experiments. The air flow rate was held constant and equal to 1.25 L/min (0.5 VVM). A minimum dissolved oxygen concentration (pO_2) of 40% was used during all experiments to ensure non-oxygen limited growth. Four kinds of impeller (see figure 1) of 0.08 m diameter were used at stirring rates between 800 and 1700 rpm and cultures were continued to give final biomass concentrations between 4 and 8 g/L, as detailed in [2].

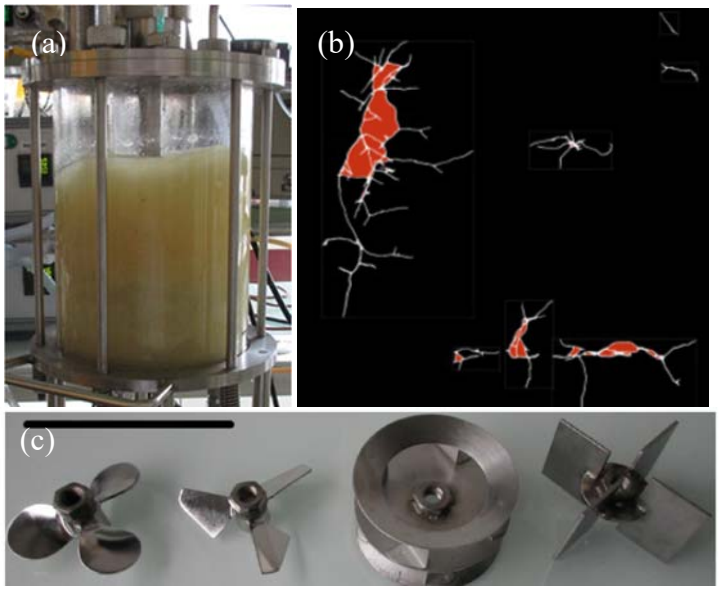


Figure 1: Experimental set-up: 3.5L bioreactor (a), Image processing to characterize morphology (b), and the different impellers used during the study(c).

The rheological properties of the broth were determined during culture by a rotational rheometer (TA Instruments, AR2000) equipped with a helical rotor. The apparent viscosity is measured in the range of shear rates [1-100 s^{-1}]. A Herschel-Bulkley model is generally used to model the rheological properties of *T. reesei,* but in the present work only apparent viscosities at $\dot{\gamma} = 10s^{-1}$ were used. The dry weight method was used to quantify the biomass concentration, with the help of a glass microfiber filter (Whatman GF/C) with particle retention of 1.2 mm. Image analysis was carried out using a new method described by Hardy et al. (2017) [4]. This method includes an original extended depth-of-field approach, a specific segmentation and both skeleton and topological analyses. Different pertinent parameters were determined to characterize the impact of fluid dynamic stress on morphology, and it was shown that, at extreme fluid dynamic stress levels, the number of holes (nH) was statistically the best descriptor of the changing morphology. The lower the agitation intensity, the greater the number of holes (nH), with the value of nH being quantified by the 90% quantile (q90) of the distribution of the number of holes per image.

Study of the production step

At industrial scale, the production is step is a fed-batch-controlled production. But in the present work, it was preferred to study the impact of agitation on the production of enzymes by using a continuous chemostat culture. This method is known as more robust for establishing the impact of changes in one parameter, as it can be measured under pseudo steady-state conditions. In return, the metabolism of the fungi is intermediate between growth and production states, as the sugar is consumed to produce biomass and enzymes at the same time. The biomass concentration was held at 7 g/L approximately and the glucose concentration remained very low. Using the Rayneri impeller (the 3rd from the left in figure 1), after several days of cultures at 800 rpm, the stirring rate was suddenly switched to 1700 rpm. This experiment was repeated twice. A quantification of the secreted proteins concentration was assessed in the broth filtrate. This assay was performed with the DCTM Protein Assay kit (Biorad, Hercules, United States of America) with the help of a range of bovine serum albumin (BSA, 0-1.5 gL-1) according to the method of Lowry et al. (1951) [7]. The activity of the enzyme cocktail was determined globally with the filter paper method recommended by I.U.P.A.C. A proteomic analysis of the intracellular proteins was achieved by using bidimensional electrophoresis. 2D-gels were created from samples collected at the two stationary phases obtained at 800 rpm and 1700 rpm. By using image analysis, it was possible to detect protein positions that were identical within the gels and the conditions, and to quantify the intensity of staining in order to compare the two

different conditions. The differentially synthesized proteins were identified by liquid chromatography coupled to mass spectrometry. The sequences were compared to the Joint Genome Institute databases (JGI, Walnut Creek, USA) for identification [7].

Results

Effect of agitation on the growth of fungi

Increasing the agitation intensity decreased the growth rate, the viscosity of the broth and the size of fungi (nH was reduced). However, it is difficult to assess the impact of different impellers on these changes without using more precise hydrodynamic parameters. Certain characteristics of the of impellers used (i.e. power number, Po and flow number, Fl) were available from their suppliers except for the turbine on the right of figure 1, for which CFD simulations were performed to calculate them. These parameters are used to compare the relevance of 3 hydrodynamic criteria that can be possibly used to characterize the impact of fluid dynamic stress and aid scale up from laboratory scale results. The chosen parameters were: the tip speed (Vtip), the specific power input (P/V) and the more complex Energy Dissipation Circulation Function (EDCF). The latter is defined as the maximum specific energy dissipation rate in the tank, ε_{max} divided by the recirculation time, i.e. the average time between 2 passages through the stirring area. The initial EDCF concept [5] has been modified here, determining ε_{max} from the relationship: $\varepsilon_{max} = 1.04 \times \rho \times Po^{\frac{3}{4}} \times N^3 \times D^2$ (Grenville and Brown, 2012) [6]. The recirculation time was calculated as the ratio between the volume of the bioreactor and the pumping flow rate. In addition to lab-scale experiments, industrial data are used for validation of the 3 tested parameters. The geometrical characteristics of the industrial bioreactor cannot be described fully for confidential reasons, but it has been characterized in term of tip speed, power input and EDCF. Figure 2 presents some important results concerning the growth step. All experiments are reported on the figure whatever the scale of bioreactor and the kind of impeller. On the left side, one can observe the correlation between the growth rate and the size of fungi (as measured by number of holes per fungi); the smaller the fungi, the lower the growth rate as the fungi is exposed to very high fluid dynamic stress. Similar results (data not shown) indicate that the decrease of size also decreases the viscosity. On the right, the 3 tested parameters (called P) are reported versus the growth rate measured during experiments. Associated determination coefficients R^2 are also reported. From this figure, it is seen that the best results are obtained with EDCF as the hydrodynamic stress parameter. The same conclusions are obtained when viscosity or morphology is investigated: the EDCF criterion is better than Vtip and P/V for quantifying the effect of agitation and its scale-up.

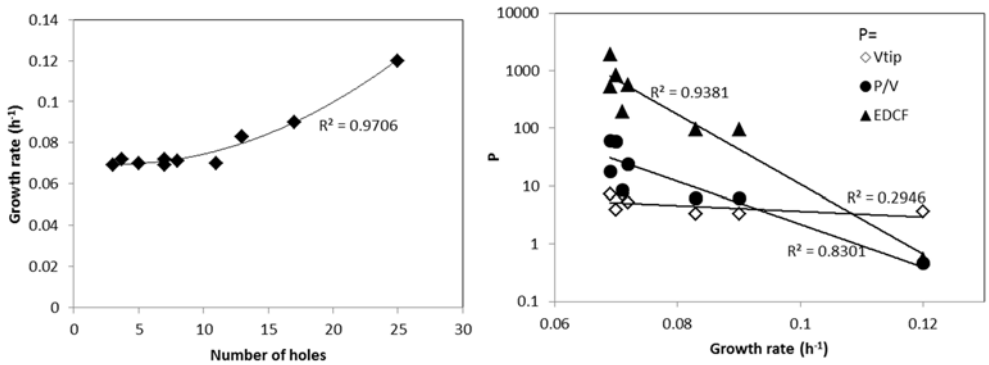


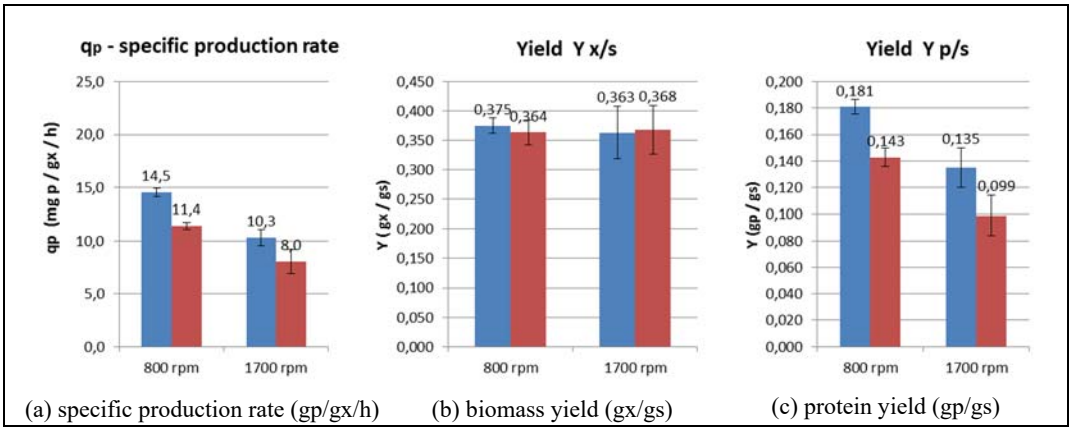
Figure 2: Link between morphology of fungi and growth rate (left); Comparison between growth rate, tip speed, power input and EDCF εmax parameters (right)

These results are in agreement with those of Justen et al. (1996) concerning the fungi *Penicillium chrysogenum* [5], and give further support to the physical meaning of the EDCF regardless of scale, the only criterion that is based on the intensity of the fluid dynamic stress close to the impellers, and the frequency of passage through that region.

Effect of agitation on enzyme synthesis

Figure 3 shows that protein production is negatively affected by higher fluid dynamic stress. showing a decrease of 30% in the specific production rate and in the production yield, when the EDCF, is increased from 157 kW/m^3/s (800 rpm) to 3200 kW/m^3/s (1700 rpm). These differences

were observed while the biomass yield was not affected, thus indicating a negative effect of high fluid dynamic stress on the physiological state of the fungi.



(a) specific production rate (gp/gx/h) (b) biomass yield (gx/gs) (c) protein yield (gp/gs)

Figure 3: Comparison of the specific production rate, biomass and protein yield for two continuous culture of Trichoderma reesei subjected to two stirring speeds (800 rpm and 1700 rpm). Experiments were performed at biomass concentrations of 7.7 g/kg (blue) and 6.1 g/kg (red)

Proteomic analysis revealed that about 40 proteins were differently or newly synthesized in the two conditions. The results indicated that when the fluid dynamic stress increased, some proteins involved in the central metabolism were over-synthesized and some stress proteins were newly synthesized, whereas proteins implicated in the production of cellulases and in less essential functions were under-synthesized. By showing a decreased production of intracellular cellulases in stressed cells, these results confirm the decrease observed in the culture broth.

Conclusions

In this study, the effect of agitation on growth rate and enzyme production has been investigated during cultures of the filamentous fungi, *T. reesei*. The induced fluid dynamic stress affects all measured process parameters: rheology, morphology, growth rate and enzyme yield. The comparison between cultures performed with different impellers at the bench scale, has shown that the effect of agitation and impeller choice on these process parameters is best correlated with the EDCF criterion. This result is validated as the EDCF also predicts rather well the process performance of a large industrial scale culture. Concerning enzyme production, it was demonstrated that high levels of fluid dynamic stress led to a decrease of protein concentration in the medium and of the intracellular cellulase production.

As the calculation of the EDCF criteria is based on the power number (Po) and flow number (Fl) of an impeller, one can show that, for a given specific power input P/V (which is dictated by the mass transfer requirement, whatever the technology used), EDCF is proportional to $D^{-2/3}(Fl / Po^{7/12})$, where D is diameter of the impeller(s). As a consequence, damages to *T. reesei* can be minimized using large impellers of high Po/Fl ratio under aerated conditions, a family of impellers improperly classified as “High Shear Impellers” by mixing equipment suppliers.

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