Experimental Guidelines to Optimize Two Crucial Steps of Lignocellulosic Bioethanol Production: A Rheological Approach

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Abstract: This paper focuses on two crucial steps of lignocellulosic bioethanol production. The first part deals with cellulases production by the filamentous fungus *Trichoderma reesei*. The second one is dedicated to the use of these enzymes on pretreated wheat straw and miscanthus to hydrolyze them into fermentable sugars. Both items have been studied using a rheological approach. Experimental results confirm that the suspension of growing *T. reesei* cells exhibits complex flow properties with yield stress and shear thinning. They usually fit with classical rheological models such as Hershel Bulkley or power law ones. This study also demonstrates that the time dependence should be considered with appropriate thixotropic model. As for the enzymatic hydrolysis , rheological tests allow to screen a large range of operating parameters such as temperature, shear rate, solids content and enzyme loading.

Keywords: Bioethanol, cellulases, lignocellulosic biomass, rheology

1 Introduction

Lignocellulosic biomass (woody crops, agricultural residues) is claimed to be one of the most significant renewable and sustainable green energy. It can be found worldwide, is abundant and can be used to produce bioethanol that does not compete with food production. The implementation of second generation biofuels is challenged by the complex structure of the lignocellulosic biomass that limits enzyme accessibility and efficiency to transform them into fermentable sugars. Actually, it is composed of cellulose, hemicellulose and lignin whose structural assembly is specific to each variety. Their complete bioconversion to ethanol

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requires several steps: pretreatment, specific enzymatic hydrolysis by cellulases, fermentation and purification [1]. They all have to be optimized in order to make lignocellulosic ethanol production cost effective. This work focuses on the hydrolysis process and aims at selecting suitable operating conditions. The paper is divided into two main parts. The first one deals with the cellulases production by a filamentous fungus: *Trichoderma reesei*. It is the most widely used microorganism at industrial scale to produce cellulases because of its high secretion capacity. The second part is dedicated to the study of the biomass hydrolysis step using these enzymes. Both issues have been studied with a rheological approach. Actually rheology is a very effective tool to provide comprehensive information about any process: it enables to study the flow of material under various physicochemical or hydrodynamic conditions (pressure, temperature, shear rate, extension, laminar and turbulent flows). It is handy to use, not time consuming and only a small volume of sample is required.

Rheology has been previously used at IFPEN in order to formulate model fluids able to mimic the cultivation of filamentous fungi with the same rheological properties. Thereby, important effect of rheology on mass transfer and mixing has been pointed out and correlations have been developed for scale-up and design of industrial bioreactors [2]. It also has been validated that mass transfer is similarly affected by apparent viscosity in model fluids and real fermentation broths of similar rheologies [2].

2 Materials and Methods

- Cellulase production : *Trichoderma reesei* strain CL-847 [3] has been used throughout this work. Enzyme productions were performed in 3 L working volume fermentors, using fed-batch technology as previously described [4–6].
- Enzymatic hydrolysis: The lignocellulosic biomass used is pretreated wheat straw and miscanthus with dilute sulphuric acid at high temperature to break down the native woody structure as described by [5].

Figure 1 Chosen geometries for the study: (**a**) 4cm roughened plate and plate, (**b**) 6cm cone and plate, (**c**) coaxial cylinders, (**d**) helicoidal ribbon.

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- The controlled stress rheometer AR2000 from TAInstrument was used with different geometries (cf. Figure 1). They were chosen depending on the product to be tested taking into account the size of dispersed phase, wall slip and settling problems. Different protocols were applied and are detailed in the main text.

3 Cellulases Production

Cellulolytic enzymes can be industrially obtained by the hyperproducing strain *Trichoderma reesei*. The fungus is first grown with sugar in excess, in order to produce a large concentration of cellular biomass during 30–50 hours. Then an inductive substrate is provided at a limiting rate to produce cellulases [7]. It is a filamentous fungus that constitutes a heterogeneous medium with complex flow properties to handle and anticipate. The enzymes quality and yield strongly depend on these rheological characteristics as they are key parameters to ensure mixing, heat and mass transfer [2]. The understanding and controlling of this phenomenon is all the more crucial that the enzyme production is a major cost of the second-generation bioethanol process.

A systematic study was undertaken on ex situ samples taken from the lab bioreactor at different time intervals. Flow curves were immediately determined by increasing shear rate from 0.1 to 1000s-1 within 3 minutes at 27°C. The shear rate range covers the ones implemented industrially. The short duration of tests limits the artifacts due to settling of dispersed solid material. The shear rate sweeps were performed with the 6cm cone plate. This configuration was chosen because it ensures well controlled conditions with a uniform shear rate within the entire volume of sample. Several tests were repeated with a 4cm roughened plate and plate using various gaps. Converging results were obtained with both geometries, which demonstrated that wall slippage was limited. Results are plotted in Figure 2.

They show that the flow behavior of the broth evolves regularly with process time. The preculture after inoculation is Newtonian and has a viscosity close to

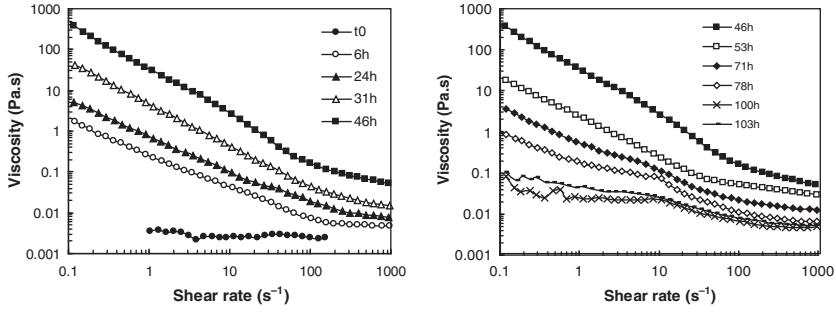

Figure 2 Flow curves of *T. reesei* broth at 27°C, cone plate AR2000 rheometer.

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the one of water. Throughout the entire growth phase, the viscosity keeps increasing and the sample develops shear thinning characteristics. This is related to the lengthening of the filaments and to their branching [8]. These combined effects lead to the formation of an overlapping network with non newtonian behavior. End of the growth phase is a critical step in the process, as apparent viscosity reaches its maximum value. Such peak of viscosity strongly affects mass transfer at the worse time, i.e. when required oxygen transfer rate is as its maximum [2]. For this reason, characterization of rheology during growth phase is critical for the choice and design of stirring impellers and associated stirring engines.

Reversely, as soon as submitted to restrictive conditions (here after 46 hours), the viscosity decreases gradually down to its initial value and to a Newtonian behavior. These rheological changes are due to the shift from an entangled structure to dispersed pellets and to a fragmentation of the freely dispersed mycelia [9]. At this point, cellulases are secreted. All the previous cultures have been filtered and the rheology of their suspending medium has been determined using the same protocol of fast shear rate sweep. For all of them a Newtonian viscosity close to 1Cp has been obtained. This indicates that during the entire fermentation process the flow properties of the broths are governed by the morphology of the dispersed fungi and not by the continuous medium.

Shear stresses have been plotted as a function of shear rate for representative times of growth and depletion steps (cf. Figure 3). They reveal that at the early beginning of the fungal development (t<31hours), the suspension gains rapidly a yield stress below which it cannot move. It confirms that the growing fungus forms a 3D connected network flowable only after a certain threshold. This feature is particularly detrimental to the process performance as it will lead to dead zones of unsheared products within the bioreactor. These static areas may also result in settling and deposit problems. In the latter part of growth (31hours<t<46hours), the suspension exhibits an even more complex behavior with a minimum in its flow

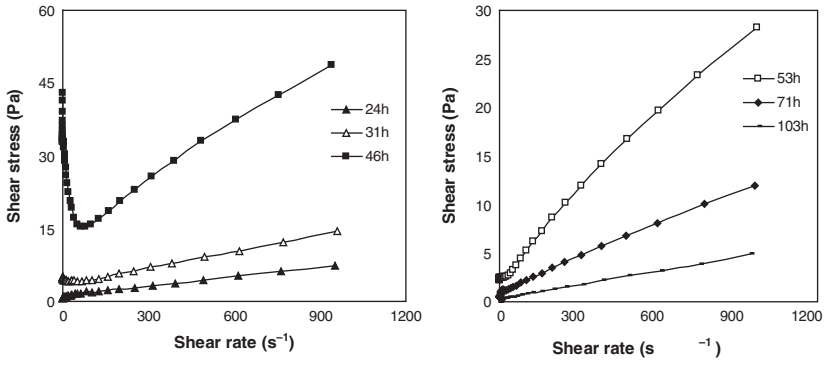

Figure 3 Yield stresses of *T. reesei* suspension during fermentation time.

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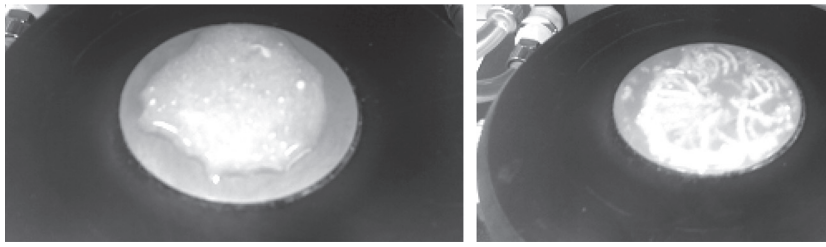

Figure 4 (**a**) initial broth, (**b**) induced shear structure.

curve. It is typical of a shear induced sandwich structure showing an unstable transition from a solid to a liquid state [10–12]. A picture of the broth before and after shear clearly shows the shear induced particular organization (cf. Figure 4). It does not appear at the early beginning of the growth when biomass concentration is low nor in the secretion step. These experimental results illustrate how complex the intermingling morphology of filamentous can be and how it may affect flow properties causing mass transfer difficulties, poor mixing and high production costs. In the nutrient limitations step, these negative effects come to a end quite quickly.

Oscillatory tests were also conducted on the fungal suspension at the height of its growth (46h). The sample was submitted to a sinusoidal strain with an amplitude of 0.04% in the frequency range of 0.1–100Hz. The very small strain allowed not to destroy the structure of the sample and to characterize it. These measurements are used to quantify the relative importance of solid and liquid properties of a viscoelastic material by distinguishing its in and its quadrature phase responses to dynamic loads. The following two oscillatory modulus are determined:

- the in phase storage modulus G′ is a measure of the energy stored and represents the elastic behavior,
- the quadrature phase loss modulus G″ is a measure of the energy dissipated and represents the viscous component.

Both modulus are compared in Figure 5. The graph confirms that the elastic behavior dominates the viscous one and that the broth exhibits a gel character.

Previous rheological tests were performed using a 6cm cone and plate that ensures accuracy and constant shear rate within the sample. The main disadvantage is that the sample tends to partly expel from it when high shear rate is applied. To avoid such a problem, a bob and cup device was employed instead of the cone and plate although shear rate is no more uniform. The so called Couette geometry was used to perform up and down sweeps on *Trichoderma reesei* broth at specific times of process (cf.Figure 6). To limit settling artefacts, each step lasted only 3 minutes. The growing filamentous fungus presents an hysteresis loop due

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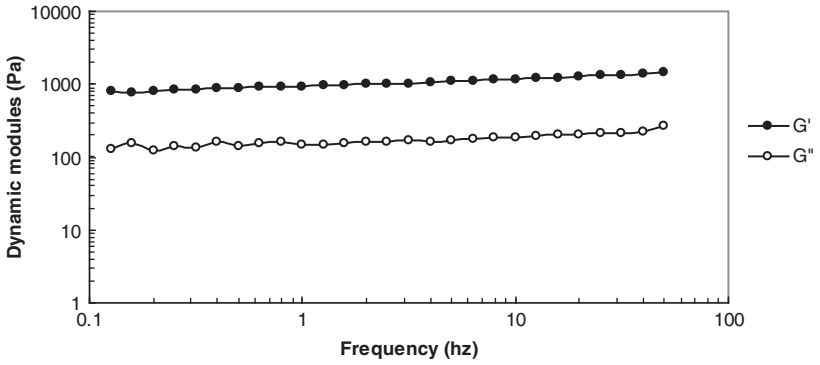

Figure 5 Dynamic modulus of *T. reesei* after 46h fermentation, cone and plate rheometer, T =2 7°C.

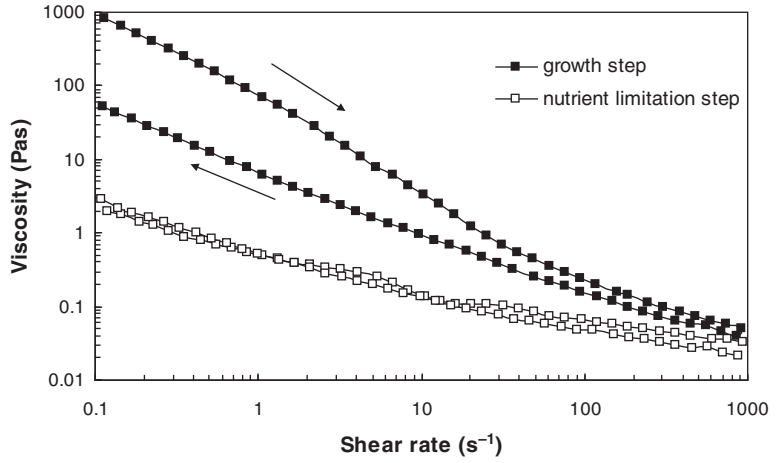

Figure 6 Time dependent behaviors of *T. reesei* in growth (thixotropy) and nutrient limitation (no thixotropy) steps.

to the decrease in viscosity with time. This thixotropy is inherent to the induced shear structure that relaxes very slowly when the shear rate decreases. The kinetics of this phenomenon will be further investigated by applying a transparent cone and plate and by varying the duration of tests. As already said, when submitted to sugar and oxygen limitations, the fungus is known to be more fragmentized (cf Figure 7). This morphology leads to a decrease in viscosity and to the absence of thixotropy: the up and down flow curves fall down and superimpose.

Based on all rheological results, the growth of *T. reesei* seems to be the crucial step that leads to the most complex flow properties. The filamentous fungi continuously change with increasing viscosity, yield stress and shear thinning behavior.

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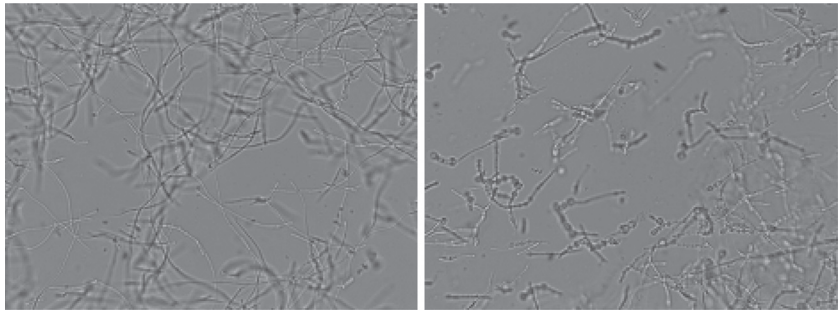

Figure 7 (**a**) Optical microscopy of growing *T. reesei* (long filaments); (**b**) under nutrient depletion (fragmentation of the freely dispersed mycelia filaments), at × 40 magnification and width of image represents approximately 1000 µm.

All these characteristics are integrated using the Hershel Bulkley model. Anyhow, we revealed that the fungal suspension is also thixotropic and that rheological models including time dependence should be considered [13].

4 Enzymatic Hydrolysis of Lignocellulosic Biomass

The process conversion of lignocellulosic biomass into fermentable sugars faces operational and economic challenges. The valuable cellulose being embedded within a complex organization of lignin and hemicellulose, the biomass to ethanol process requires technical adjustments. As already mentioned in introduction four main steps are necessary: physico-chemical pretreatment, enzymatic hydrolysis, fermentation and distillation [1] and represent significant costs. The final ethanol titer has a strong positive effect on the processing costs. Performing the enzymatic hydrolysis step at high solids concentration allows a strong reduction of the free liquid water thus increasing the concentration of sugar and consequently of ethanol. But a high solids content is also correlated with a decrease of the hydrolysis yield due to several factor such as product inhibition, cellulase adsorption on lignin or substrate composition. The optimal % of water-insoluble solids WIS is directly dependant of the operating conditions and may be higher than 12 % [14].

In order to optimize operating conditions of the enzymatic hydrolysis step, a rheological approach was proposed. An enzymatic hydrolysis was performed within the rheometer on real pretreated wheat straw and miscanthus). This experimental approach allowed to follow the decrease of the apparent viscosity of the biomass during the hydrolysis of the cellulose by the enzymes under controlled physicochemical and hydrodynamic conditions. It also minimized the amount of enzymes needed to perform enzymatic hydrolysis and optimized several critical parameters such as WIS content, temperature and shear rates. The previous bob and cup device could not be employed due to its narrow gap not suitable for slurries. A calibrated helical

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impeller was selected to avoid this problem. The use of parallel plate is prevented because enzymes cannot be introduced during trials. It also has the inconvenient of squeezing out interstitial liquid during gap setting due to applied compressive forces [15]. Peak hold steps of constant shear rate were applied.

Consistently, samples of miscanthus at 20% of WIS miscanthus were prepared in a 50mM acetate buffer at pH 4.8. They were introduced into the rheometer and held until a constant temperature was reached. Then a complete enzymatic cocktail from *T. reesei* enzyme was poured under stirring and its activity was measured by the resulting drop in viscosity. The impacts of enzyme loading, temperature and shear rate were evaluated by comparing the kinetics of decrease and its extent.

The first series of liquefactions were realized at 45°C, under $20s^{-1}$ with different enzymes loading ranging from 2.5 to 20mg/g of WIS. Results are shown in Figure 8 by plotting the ratio between the viscosity in the course of time η_t and the viscosity at the end of the enzyme pouring η_0. As expected, the hydrolysis reaction gets faster with the highest amount of cellulases. The responses are quite different and confirm that rheology can provide sensitive information on bioprocess.

The second series of liquefactions were realized at $20s^{-1}$ with 20mg of enzyme per g of WIS at various temperatures: 35, 45 and 55°C. Their comparison is presented in Figure 9. Liquefaction is accelerated by high temperature but the beneficial effect seems to reduce after 45°C. This temperature appears as a good compromise between enzyme efficiency and energy consuming.

The third series of liquefactions were realized at 45°C with 20mg/g of enzyme at various shear rates: 10, 20 and $30s^{-1}$. Their comparison is presented in Figure 10. Liquefaction does not seem to be affected by shear rate. One possibility for cost savings might be to gently decrease agitation level.

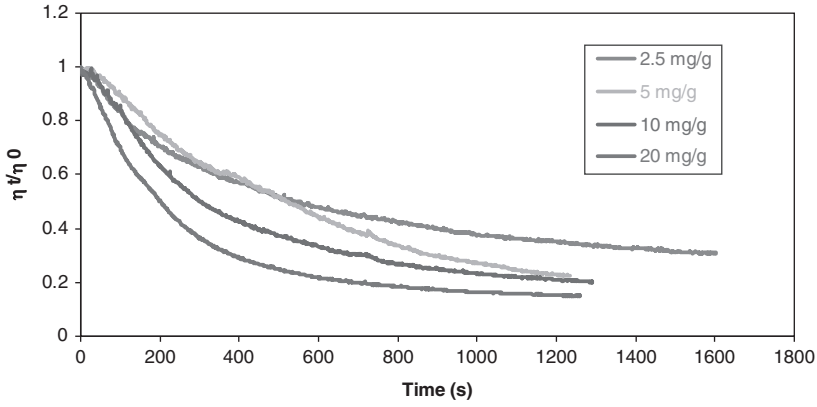

Figure 8 Influence of enzyme dosage on liquefaction kinetics.

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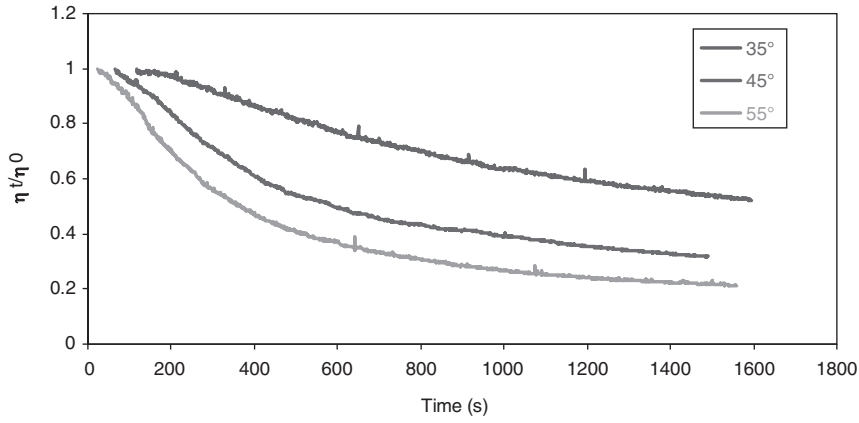

Figure 9 Influence of temperature on liquefaction kinetics.

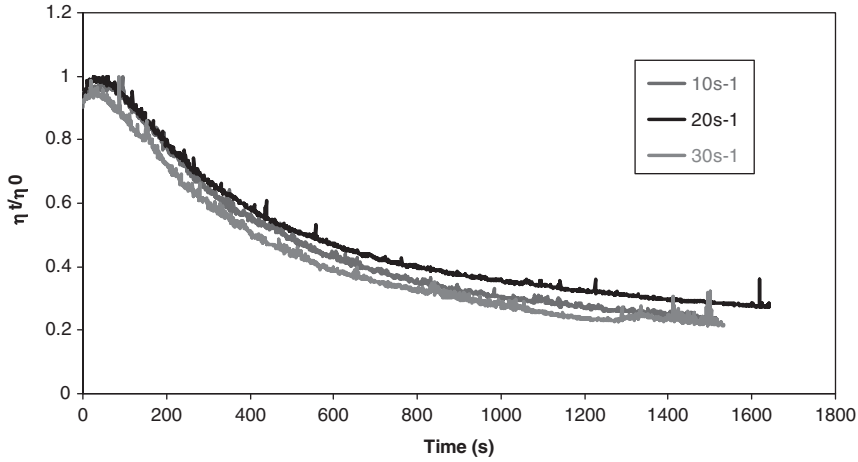

Figure 10 Influence of shear rate on liquefaction kinetics.

5 Conclusion

Experimental results confirm that the suspension of growing *T. reesei* exhibits complex flow properties with yield stress and shear thinning. They usually fit with classical rheological models such as Hershel Bulkley or power law ones. This study demonstrates that the time dependence should also be considered with appropriate thixotropic model. Rheological studies of enzymatic hydrolysis at lab-scale allow to screen a large range of operating parameters such as temperature, shear rate, solids content and enzyme loading. As the optimal operating conditions is also

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dependant of the industrial enzymes used (% of cellulolytic and hemicellulolytic activities) and the physico-chemical properties of the pretreated substrates (% cellulose, quality of the lignin, % residual hemicellulose etc.), this tool will be helpful for the standardization of the operating conditions of the saccharification step. it also offers the possibility to develop new enzymatic cocktail dedicated to the liquefaction phase by testing reconstituted enzymatic cocktails using purified enzymes from *T. reesei* industrial preparation.

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References

1. N. Mosier, C. Wyman, B. Dale, R. Elander, YY. Lee, M. Holtzapple and M. Ladisch, Features of promising technologies for pretreatment of lignocellulosic biomass'. *Bioresour. Technol*. **96**, 673–686 (2005).
2. J.C. Gabelle, E. Jourdier, R. Licht, F. Ben Chaabane, I. Henaut, J. Morchain and F. Augier. Impact of rheology on the mass transfer coefficient during the growth phase of *Trichoderma reesei* in stirred bioreactors. *Chem. Eng. Sci.* **75**, 408–417 (2012).
3. H. Durand, H., Clanet, M. and Tiraby, G. Genetic improvement of *Trichoderma reesei* for large scale cellulase production. *Enzyme Microb. Technol.* **10**, 341–346 (1988).
4. J. Pourquié, M. Warzywoda, F. Chevron, D. Longchamp, and J.P. Vandecasteele, Scale-up of cellulase production, in FEMS Symposium No.43: Biochemistry and Genetics of cellulose (1988).
5. M. Warzywoda, E. Larbre, J. Pourquie. Production and characterization of cellulolytic enzymes from *Trichoderma reesei* grown on various carbon sources. *Bioresour. Technol*. **39**, 125–130 (1992).
6. E. Jourdier, L. Poughon, C. Larroche, F. Monot and F.B. Chaabane. A new stoichiometric miniaturization strategy for screening of industrial microbial strains: application to cellulose hyper-producing *Trichoderma reesei* strains. *Microb. Cell. Fact* **11**, 70 (2012).
7. F. Ben Chaabane and R. Marchal. Upgrading the Hemicellulosic Fraction of Biomass into Biofuel. *Oil & Gas Science and Technology–Revue d'IFP Energies nouvelles* (2013).
8. P. Znidarsic and A. Pavvko The morphology of filamentous fungi in submerged cultivations as a bioprocess parameter. *Food Technol. Biotechnol.* **39**, 237–252 (2001).
9. A. Amanullah, L.H. Christensen, K. Hansen, A.W. Nienow and C.R. Thomas, Dependence of morphology on agitation intensity in fed – batch cultures of *Aspergillus oryzae* and its implications for recombinant protein production. *Biotechnol. Bioeng*. **77**, 815–826 (2002).
10. P.H. Coussot, A.I. Leonov and J.M. Piau, Rheology of concentrated dispersed systems in a low molecular weight matrix. *J. Non-Newtonian Fluid Mech.* **46**, pp 179–217 (1993).
11. R. Mas and A. Magnin, Rheology of colloidal suspensions: case of lubricating greases. *J. Rheol*. **38**, 889 (1994).

DOI: 10.7569/JSEE.2013.629524

12. F. Pignon, A. Magnin and J.M. Piau, Thixotropic colloidal suspensions and flow curves with minimum: Identification of flow regimes and rheometric consequences. *J. Rheol*. **40**, 537–587 (1996).
13. J. Mewis and N.J. Wagner, Thixotropy, Advances in colloid and interface sciences, **147–148**, pp 214–227 (2009).
14. A. Wingren, M. Galbe and G. Zacchi. Energy considerations for a SSF-based softwood ethanol plant. *Bioresour. Technol.* **99**, 2121–2131 (2008).
15. J.S. Knutsen and M.W. Liberatore, Rheology of high-solids biomass slurries for biorefinery applications. *J. Rheol*. **53**, 877–892 (2009).

DOI: 10.7569/JSEE.2013.629524