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Steady and Dynamic Shear Characterization of Cellulase-Producing *Trichoderma reesei* Suspensions

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ABSTRACT

Suspension rheology of fungal fermentations is important in determination of mass transfer rates, as well as mixing quality. We have characterized *Trichorderma reesei* RUT-C30 suspension rheology during growth on xylose (soluble) and cellulose (particulate) substrates, using both steady and dynamic shear measurements. Biomass growth was monophasic on xylose and biphasic on cellulose; the latter behavior is consistent with relatively rapid, early growth on soluble sugars derived from rapidly hydrolyzed material, followed by a second, slower growth phase owing to hydrolysis of more recalcitrant cellulose by increasing cellulase concentrations.

Steady shear measurements established the presence of a yield stress for fermentation broths when using a 10 (vol)% fungal inoculum. The Casson equation represented all data well. Casson parameters of viscosity and yield stress followed biomass evolution: two maxima in both parameters were observed with cellulose substrates, and a single maximum with xylose. Dynamic shear measurements on broths indicated a gel behavior at small strains and a shear thinning liquid behavior at larger displacements. These results indicate the need to include rheology and mixing considerations in the subsequent development of a full biological and physical kinetic description of *T. reesei* cellulose conversions.

Index Entries: Rheology; *Trichoderma reesei*; fermentation; steady shear; dynamic shear.

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INTRODUCTION

The rheology of *Trichoderma reesei* suspensions during cellulose hydrolysis and cellulase production has been little studied, yet rheology is crucial in establishing oxygen gas–liquid mass-transfer conductances and power requirements for both mechanical mixing and aeration (*1*). Several researchers have studied the rheological behavior of other fungal broths under steady shear conditions (*1–5*). The two constitutive equations most often used to represent this data are the Casson model given by Eq. (1):

$$(\tau)^{1/2} = (\tau_y)^{1/2} + K_c\,(\dot{\gamma})^{1/2} \tag{1}$$

where τ_y is the Casson yield stress and K_c is the Casson viscosity, and the power law model given by Eq. (2):

$$\tau = K(\dot{\gamma})^n \tag{2}$$

where K is the power law consistency and n is the power law index.

As part of a larger study seeking development of a full description of both biological kinetics (substrate consumption, biomass growth and reduction, and product formation) and physical kinetics (oxygen mass transfer and suspension mixedness), we have first developed a vane-and-cup rheometer cell for off-line analysis of fungal broth rheology. We use it to establish correlations between the rheological parameters appropriate to steady shear and dynamic shear models and the causative biomass levels in the suspension. A summary of procedures and key results follow; a full report is published elsewhere (*6*).

MATERIALS AND METHODS

T. reesei RUT-C30 spores were converted into inocula using production media described earlier (*7*) with slight modifications (*8*). Successive 50-mL and then 1000-mL inocula were grown on 10 g/L glucose and 10 g/L cellulose (Sloka floc 200FCC, 35 μm fiber length, Fiber Sales and Development Corp., Urbana, OH), respectively, at 28°C on an oscillating shaker (225 rpm).

Fermentation Conditions

A 16-L, steam-sterilizable fermentor (Microgen SF116, New Brunswick Scientific, Edison, NJ) equipped with three equally spaced Rushton impellers (D_i/T = 0.35) was loaded with 9 L medium and 1 L inoculum, and cultured at 28°C, impeller speed of 250 rpm, with air sparging = 0.2 – 1.0 vol/vol/min, and pH = 4.8 maintained by acid (H_3PO_4, 3*M*)/base (NH_4OH, 3*M*) addition. Dissolved oxygen (DO) concentration in the broth averaged 35 ± 15%, except under extreme growth when DO fell

below 20%. Addition of antifoam 289 (Sigma Chemical) prevented foaming, whereas addition of penicillin prevented contamination.

Analysis Procedures

Total (mycelium and cellulose) dry weight was determine by filtering 10 mL of culture broth, washing with an equal volume of water, and drying at 55°C for 24 h. Mycelium dry weight was determined via modified Lowry method (total cell-protein assay) (*8*) with bovine serum albumin (BSA) standard. The assay was calibrated with broth from xylose (cellulose-free) grown culture. For this assay, 1 mL of broth sample was centrifuged at 13,600*g* for 5 min, washed with an equal volume of water, and stored at −20°C for later analysis. Free cellulose concentration was calculated as the difference between total dry weight and mycelium dry weight. Cellulase activity, as IFPU/mL, was determined as per the International Union of Pure and Applied Chemistry (IUPAC) recommendation (*9*). Reducing sugars were determined by the dinitrosalicylic acid (DNS) method (*9*).

Rheological Apparatus and Procedure

All rheological measurements were performed off-line, in a Mechanical Spectrometer (RMS-800, Rheometrics, Inc., Piscataway, NJ) fitted with a vane-and-cup rheometer cell (vane: 4 blades 90° spacing, diameter = 35.7 mm; cup: diameter = 47.6 mm, height = 60.2 mm), which is more useful for handling particulate suspensions than are conventional cone-and-plate or concentric cylinder narrow gap cells (*2,10,11*). At various times during fermentations, a 120-mL sample was removed from the fermenter; 30 mL were prepared for later analytical analysis, and 90 mL were immediately loaded into the cup for rheological analysis. The cup was mounted in the rheometer, and the vane was lowered into the sample so that the top of the vane was even with the liquid level. Several rheological tests were run sequentially. The ranges of conditions examined were:

Steady shear:	$0.1 < \dot{\gamma}(s^{-1}) < 10.0$
Dynamic shear:	frequency sweep (at 1% strain): $0.1 < \omega(\text{rad/s}) < 20$
	strain sweep (at 4 rad/s): $0.1 < \gamma(\%) < 100$

Rheometer Calibration for Steady Shear Experiments

The average shear rate, $\dot{\gamma}_{avg}$, and average shear stress, τ_{avg}, in an impeller or vane mixed-system may be expressed in terms of dimensionless geometric constants (c, k), system dimensions (D_i = impeller diameter, cm), and operating conditions (N = impeller rotation rate, s^{-1}; M = shaft torque, dyn·cm) (*2*) according to Eqs. (3) and (4):

$$\dot{\gamma}_{avg} = kN \tag{3}$$

$$\tau_{avg} = [(2\pi k) / (cD_i^3)]\, M \tag{4}$$

The corresponding apparent viscosity is given by Eq. (5):

$$\eta \equiv (\tau_{avg} / \dot{\gamma}_{avg}) = (2\pi M) / (cN\, D_i^3) \tag{5}$$

Following calibration procedures outlined in the literature (2,4), we used a Newtonian fluid (silicon fluid 200/500, Dow Corning, Midland, MI) and a non-Newtonian fluid (xanthan gum) to obtain the following geometric constants for our vane-and-cup system:

$$c = 211.91 \pm 0.02 \tag{6}$$

$$k = 12.48 \pm 0.02 \tag{7}$$

Dynamic Rheometry

Dynamic oscillatory shear measurements utilize application of a sinusoidal deformation, $\gamma(t) = \gamma_o sin(\omega t)$, to generate a corresponding measured shear stress consisting of in-phase and out-of-phase components represented by G' and G'' (*12*):

$$\tau_{yx} = G'\gamma_o \sin(\omega t) + G''\gamma_o \cos(\omega t) \tag{8}$$

where G' and G'' are the storage (elastic) and loss (viscous) moduli of the suspension and ω (rad/s) is the frequency of oscillation. The shape and magnitude of G' and G'' reveal suspension microstructure (*12–15*). For example, a sample with $G' = 0$ and $G'' \neq 0$ is a purely viscous liquid, $G' > G''$ and independent of ω is a gel, and $G'/G'' = \infty$ is a perfect elastic solid.

RESULTS

The rheometer cell calibration was satisfactory in two key respects: (1) Log power number ($N_P \equiv P/\rho_l N^3 D_i^5$) plotted vs log Reynolds number ($N_{Re} \equiv \rho_l N D_i^2/\eta$) for our system was linear (indicates laminar flow regime), as required for evaluation of the geometric constant c: $N_P = c/N_{Re}$ (*2*). (2) The constant k was determined by comparison of steady shear rheometry between our vane-and-cup and a standard cone-and-plate geometry using a given xanthan gum solution to give $k = 12.48 \pm 0.02$. Using these k and c values, vane-and-cup measurements at diluted xanthan concentrations then provided power law parameters (consistency, K, non-Newtonian index, n) differing by only 2 and 5%, respectively, from cone-and-plate values, thus validating our vane-and-cup system.

The evolution of substrate, cell mass, and free cellulase enzyme product is shown in Figs. 1A and 2A for xylose and cellulose substrates, respectively. Figure 1A shows growth on xylose to reach a single, maximum biomass concentration. The onset of cellulase production occurred

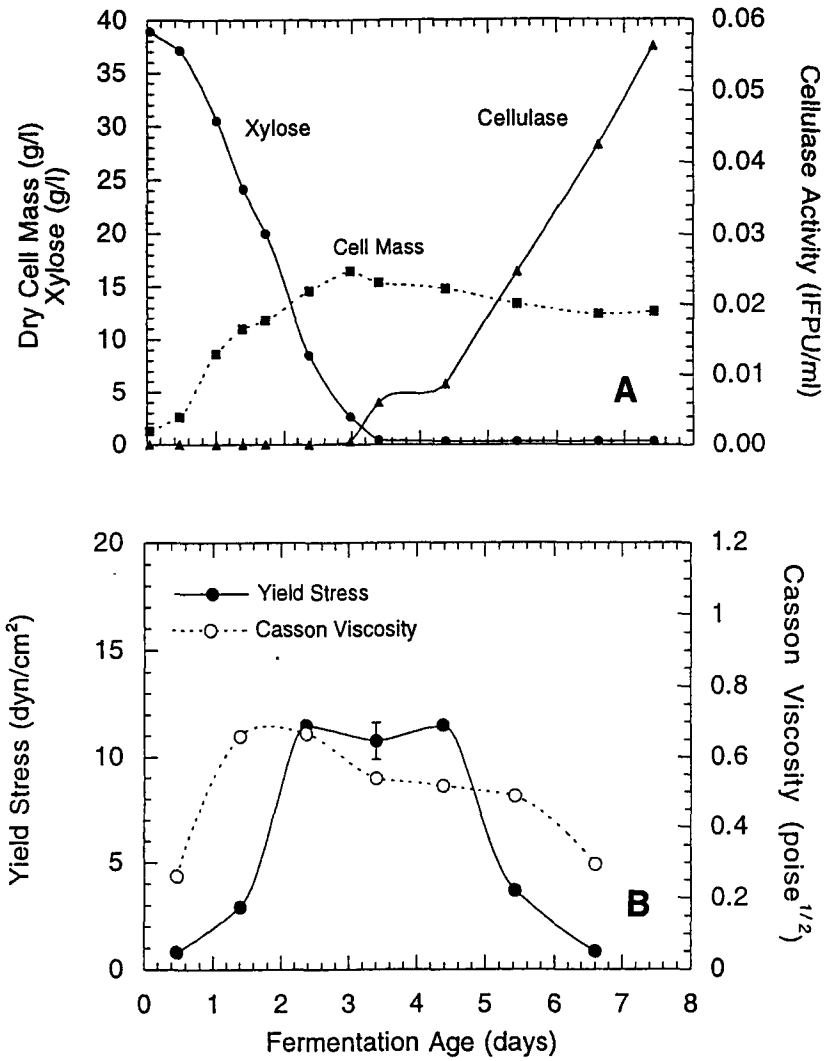

Fig. 1. Variation in (**A**) dry cell mass, cellulase activity, reducing sugar, cellulose concentration, and (**B**) yield stress (τ_y) and Casson viscosity (K_c), with time; *T. reesei* fungal fermentation with 4% initial xylose.

at xylose exhaustion. Microscopy also showed evidence of spore formation as substrate exhaustion was reached. Utilization of cellulose (Fig. 2) appeared to proceed in two stages: rapid initial growth during the first day, apparently on easily hydrolyzed cellulose, followed by slower growth and a maximum biomass level reached at cellulose exhaustion. In all runs, the rheological parameters for both steady shear and dynamic shear measurements reflected single or dual maxima for growth on xylose and cellulose substrates, respectively.

Steady shear rheometry indicated that the Casson equation [Eq. (1)] provided a much better fit for all data than did the power law (Eq. [2]).

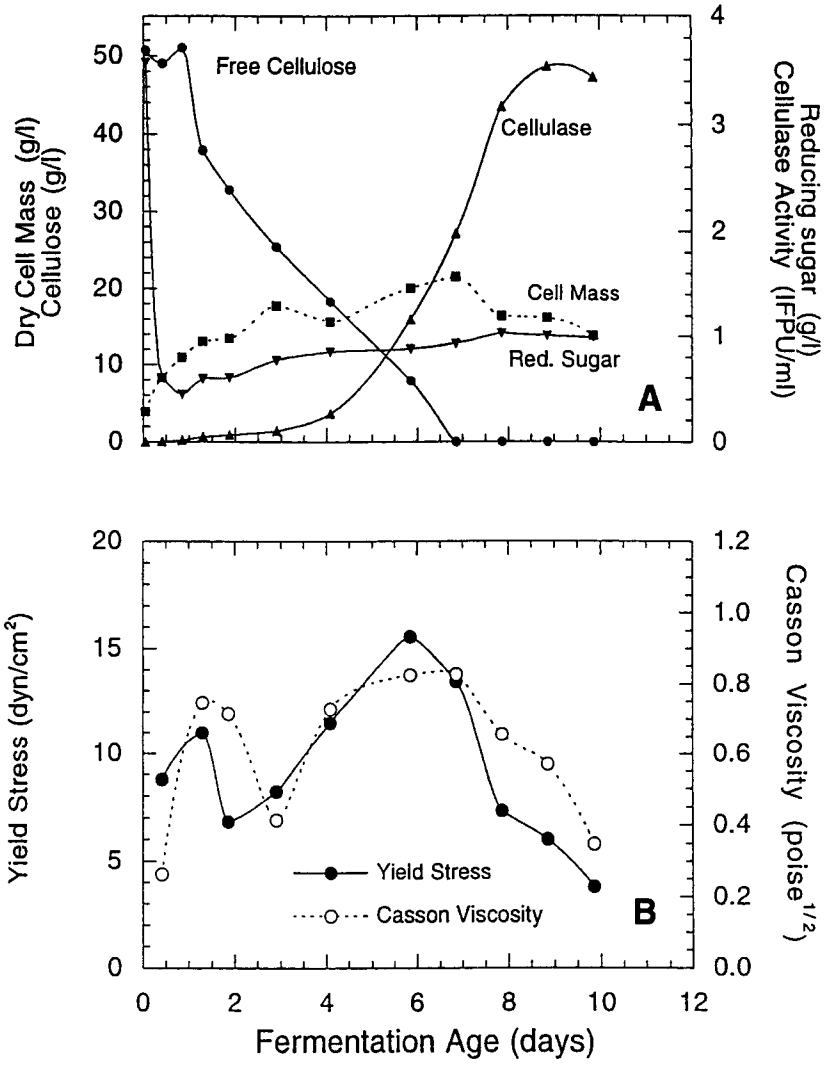

Fig. 2. Variation in (**A**) dry cell mass, cellulase activity, reducing sugar, cellulose concentration, and (**B**) yield stress (τ_y) and Casson viscosity (K_c), with time; *T. reesei* fungal fermentation with 5.0% initial cellulose.

For the xylose fermentation, the time evolution of the Casson yield stress (τ_y) and Casson viscosity (K_c) each showed a single strong maximum. Yield stress peaked at about the same time as the peak in cell mass, but Casson viscosity reached a maximum at an earlier time (Fig. 1). Cellulose conversions produced biphasic variation in yield stress and Casson viscosity (Fig. 2), with the first maximum appearing at about 2 d after disappearance of utilizable reducing sugars, and the second (day 5) again appearing just in advance of maximum biomass concentration (day 6). These behaviors strongly suggest that biomass variation is the dominant cause of rheological variations; however, the time shifts observed

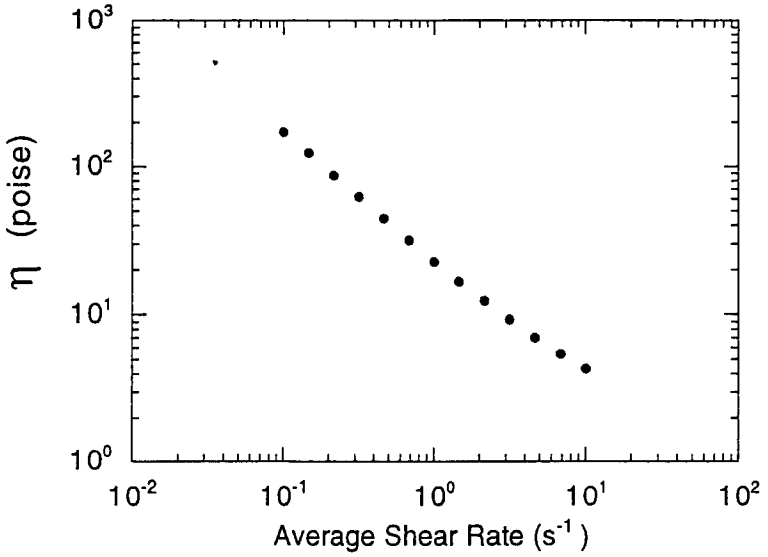

Fig. 3. Apparent viscosity (η) as a function of shear rate for a *T. reesei* fungal suspension grown on 5.0% initial cellulose. Cells were removed from fermentor 1.3 d after inoculation.

between biomass maxima and Casson model parameter maxima indicate that an additional variable, probably mycelial morphology, is also playing a role. Researchers working with other fungal systems (*16,17*) have reported morphology to influence significantly broth rheological behavior.

The strongly shear-thinning nature of these suspensions is illustrated in Fig. 3, which shows that apparent viscosity (η) falls by a factor of nearly 30 as average shear rate increases 100-fold. From Eq. (3), we calculate that the average fermenter shear rate was approx 50/s. Therefore, in traditionally configured fermenters, regions behind baffles and piping will have much lower shear rates and thus higher apparent viscosities; the possibility for substantial mixing nonuniformity is evident. We note that the cellulose suspensions themselves are reported as having low viscosity with nearly Newtonian behavior. Ang (*18*) found that powdered cellulose (same fiber length used in our study) did not appear to have any thickening properties when suspended in water at concentrations of 3.0%. With our vane-and-cup system, a 5% cellulose suspension had an apparent viscosity below the measurable range of our rheometer. Thus, the cellulose suspensions alone exhibit apparent viscosities far below those shown in Fig. 3.

The dynamic shear measurements were performed only on the 5% initial cellulose broths. The average of elastic (G') and viscous (G'') moduli at low strain values (0.1–1%) varied in a bimodal manner over the course of the fermentation (Fig. 4), with maxima corresponding exactly to the maxima measured for steady shear parameters (Fig. 2). At all such small strain measures, the ratio of elastic modulus (G') to viscous modulus (G'')

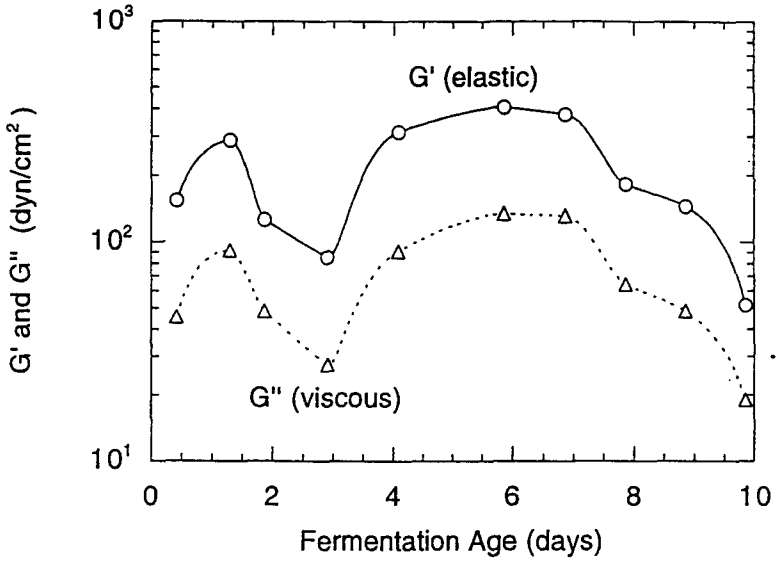

Fig. 4. Elastic (G') and viscous (G'') moduli from frequency sweeps over the course of a fermentation with 5% initial cellulose. Moduli were essentially constant over the range $0.1 < \omega$ (rad/s) < 10. Plotted values represent the average of each parameter over this range.

ranged between 3 and 4. Thus, at low strain or displacement, the cell broth behaved as a gel, consistent with steady shear rheology and indicative of a yield stress.

Dynamic shear measurements at higher strains (Fig. 5) indicate that above approx 10% strain, the dynamic moduli G' and G'' become equal. The transition from $G'/G'' \cong 3$–4 to $G'/G'' \cong 1.0$ corresponds to a shift from gel to viscous liquid behavior. Figure 5 implies that in the impeller region (high shear), the broth would behave like a liquid, whereas near poorly mixed zones, a gel-like behavior is expected. A two-region fermentation volume may result, consisting of a well-mixed liquid fraction and a poorly mixed gel region.

CONCLUSIONS

Initial characterization of *T. reesei* fermentation broths grown on xylose or cellulose shows the following behavior:

1. Biomass concentration, known to be the dominant variable in rheology of other fungal cultures (*16,17*), passes through unimodal and bimodel behavior for growth on xylose and cellulose, respectively;

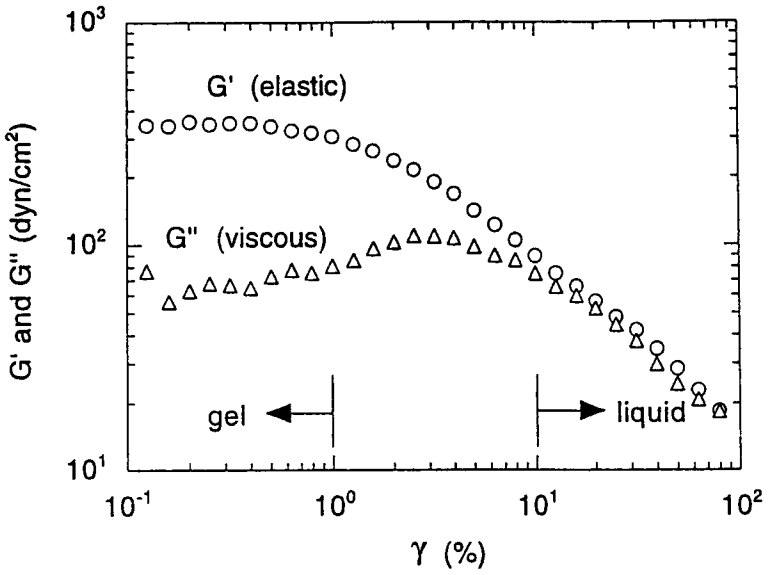

Fig. 5. Elastic (G') and viscous (G'') moduli as a function of percent strain (γ), in a typical strain sweep oscillatory shear measurement (ω = 4.0 rad/s); *T. reesei* fermentation with 5% initial cellulose, 1.3 d after inoculation.

2. A vane-and-cup rheometer cell provides adequate measures of non-Newtonian fluids (xanthan) and suspensions (*T. reesei*);
3. Steady shear measurements indicate that the Casson equation fits all *T. reesei* data well for all fermentation times when 10 (vol)% inoculum is used;
4. Dynamic shear study illustrates that 5% cellulose substrate produces a fermentation fluid that is gel-like at small strains (<1%) and liquid-like at larger strains (>10%); and
5. All rheological parameters for the steady shear Casson equation and the dynamic shear measurements exhibit maximum values at or near the maximum in biomass concentration. Biomass morphology (not reported here) may be an additional variable requiring characterization; studies on this dimension are being initiated.

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