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Use of Cellulase from *Pseudomonas fluorescens* for the Hydrolysis of Waste Paper in an Attrition Bioreactor†

Scientific Note

T. C. SCOTT,* J. M. COSGROVE, C. L. COON, J. A. KENNEY, AND C. D. SCOTT

Chemical Technology Division, Oak Ridge National Laboratory, P. O. Box 2008, Oak Ridge, TN 37831-6226

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INTRODUCTION

Typically, municipal solid waste is made up of about 48% paper, 25% glass, 13% plastics, 6% steel, 2% aluminum, and 6% other materials (*1*). The volume of this material is very large, estimated to be in excess of 180 million t/yr in the United States with newsprint alone making up about 14% of the total (2). Segregated waste-paper products could be an ideal feed material for biological conversion to sugars with the possibility of subsequent conversion to ethanol. If half of the newsprint were recycled and only half of the remaining paper products were available as a feedstock, over 30 million t/yr would be available for processing (*1*). With an 80% conversion of the included cellulose to ethanol, this would represent a potential for over 4 billion gal/yr of ethanol or nearly four times the present yearly US production.

*Author to whom all correspondence and reprint requests should be addressed.

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The primary processing steps for such a process must include:

1. Handling and size reduction of waste paper;
2. Enzyme production for use in a hydrolysis reactor;
3. Enzymatic hydrolysis of the cellulose fraction;
4. A fermentation system for conversion of hydrolyzed sugars to a crude aqueous ethanol; and
5. A means of concentrating and purifying the end product (3).

At the present time, the critical steps in this approach involve the production and use of enzymes in an effective hydrolysis reactor. One possible approach involves the simultaneous saccharification of cellulose and fermentation of the resulting glucose to ethanol (SSF) (4). This approach has the advantage of combining two of the critical processing steps into a single bioreactor. The weakness of this approach as it currently stands is that the conditions (pH, temperature, and so on) of the current enzymatic process of choice and the current fermentation process of choice are such that when run together, neither is near optimal. Continued research in this area should have prospects for future enhancements.

The approach studied in this scientific note represents an alternative to SSF. The idea is to optimize the enzymatic hydrolysis and fermentation steps separately. For waste-paper hydrolysis, our approach is to provide an attritting force that constantly creates fresh surface area for enzymatic attack. This is accomplished by using an external centrifugal pump that provides a constant shearing of the wastepaper to create solid–liquid surface area. Another important aspect of this study is the production of cellulase systems by bacteria. The overall intent is to be able to produce low-cost cellulase sources, and then use them in reactors that provide for the most efficient interaction between enzyme and solid substrate. This article describes preliminary findings for the use of a bacterial cellulase in an attrition bioreactor for the purpose of enhancing the hydrolysis of cellulose in waste paper.

MATERIALS AND METHODS

Production of Cellulase Enzymes

The bacterium identified in the literature as *Pseudomonas fluorescens var. cellulosa* (NCIB 10462) (5) was used to produce the cellulase enzymes utilized in the waste-paper studies. More detailed taxonomy of this organism has revealed that it has not been properly identified in the past. The bacterium constitutively excretes cellulase (6); hence, the enzymes can be easily recovered from the supernatant of a batch growth experiment using ultrafiltration. The cells were grown in a 75-L New Brunswick Scientific Fermentor in 50-L batches. Growth was carried out at 30°C in a Tap M-9 media that was buffered to pH 7.4 with 0.1% w/v Avicel and 0.025% w/v

carboxymethylcellulose (CMC). The Tap M-9 media was comprised of 6 g sodium phosphate dibasic, 1 g potassium phosphate monobasic, 0.5 g NaCl, 1 g NH_4Cl, 1 mL of 0.1*M* $CaCl_2 \cdot 2H_2O$, and 1 mL of 1*M* $MgSO_4 \cdot 7H_2O$ in 1 L of tap water. In these preliminary experiments, the Avicel and CMC were used as carbon sources to ensure that the bacterium had a competitive advantage for growth over potential contaminants. The system was aerated at the rate of 10 slpm with an agitation rate of 60 rpm. Runs took from 3 to 7 d to obtain cell densities of 10^{10} cells/mL.

Supernatants from these growth runs were filtered through a 0.1-μm filtration cartridge to remove cells and suspended Avicel. The permeate from the 0.1-μm filter was concentrated using a hollow-fiber ultrafiltration cartridge with a 10-kDa cutoff (Amicon). Two ultrafiltration systems (Amicon models DC30A and CH2) were utilized to concentrate the permeate from 50 L down to 500 mL. The 100X concentrated supernatant was then used as the enzyme source for the reactor studies.

Enzyme activities were measured with both CMC and Whatman's no. 1 filter paper as substrates, thus giving a measure of both CMC-ase and filterpaper-ase (FP-ase) activity. Activity was measured by placing an aliquot of the enzyme solution in contact with the substrate at a given temperature for a prescribed amount of time. These readings were carried out in Tap M-9 at a pH of 7.4. Total reducing sugars were then measured using the 3,5-dinitrosalisylic acid reagent (DNS) method (*7*). CMC-ase readings were incubated for 60 minutes with a 2% w/v solution of CMC, whereas FP-ase readings used a 1 × 6 cm strip of filter paper and were incubated at temperature for 120 min. Assuming that all of the reducing sugar measured was glucose, the activity was reported in terms of μmol glucose/min or IU. Hence, activity readings in IU are accompanied by the substrate, pH, and temperature.

Attrition Bioreactor

Figure 1 contains a schematic diagram of the reactor system used in the experiments. The reactor was a jacketed glass column with a 3.8 cm id and a length of 61 cm. A glass fitting that reduced the diameter from 3.8 to 2.5 cm was added at the top of the reactor to minimize the air/liquid interface where the waste-paper slurry entered the reactor. The slurry was continuously removed from the bottom of the column and recirculated through the attritting centrifugal pump (March Mfg., Inc., Model MDX-3). Samples were periodically taken from a side-arm sample port near the top of the reactor. Recirculation time through the system was approx 5 s. Total system volume was 1 L, 750 mL of which was contained in the glass section with the balance in the pump head and connecting lines.

Experimental Procedure

The reactor charge was comprised of a 1% w/v slurry of mixed waste paper in buffer solution containing the enzyme. Before introduction into

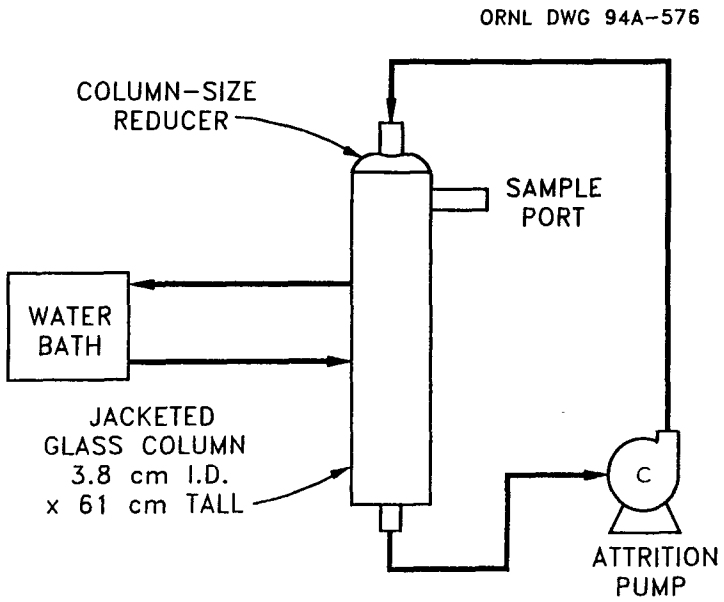

Fig. 1. Schematic diagram of the cellulose hydrolysis system.

the reactor, the paper was cut into 2.54-cm squares and blended in a Waring Blender (Model 34BL97) on setting 4 for 90 s in a 5% slurry with the reactor buffer solution. The buffer solution was Tap M-9 media at pH 7.4. The experiments were carried out at either 15 or 35°C with the pH being held constant at 7.4 by the buffer. Liquid samples were removed from the reactor as a function of time to test for total reducing sugars present from hydrolysis and for remaining CMC-ase and FP-ase activity in the liquid phase.

RESULTS AND DISCUSSION

Figure 2 contains a plot of the CMC-ase activity per gram of paper in the reactor as a function of time for three hydrolysis experiments. Hydrolysis experiments and enzyme activity measurements were all carried out at pH 7.4. Run CL3 was carried out at 15°C, whereas CL4 and CL5 were duplicate runs carried out at 35°C. It is evident that the CMC-ase activity is dependent on temperature with a significantly higher activity occurring at 35°C, as would be expected. In each case, after an initial drop, the CMC-ase activity remained essentially constant for 24 h. The same pattern is shown in Fig. 3, where FP-ase activity is plotted as a function of time. The same general trend with temperature is noted. These initial drops in activity are not surprising, since the initial samples are taken before introduction of the paper. Cellulase components are expected to undergo a certain degree of adsorption to the waste-paper substrate after introduction into the reactor; thus, the initial drop is most likely related to a combination of enzyme adsorption to the substrate and any declines that may be occurring

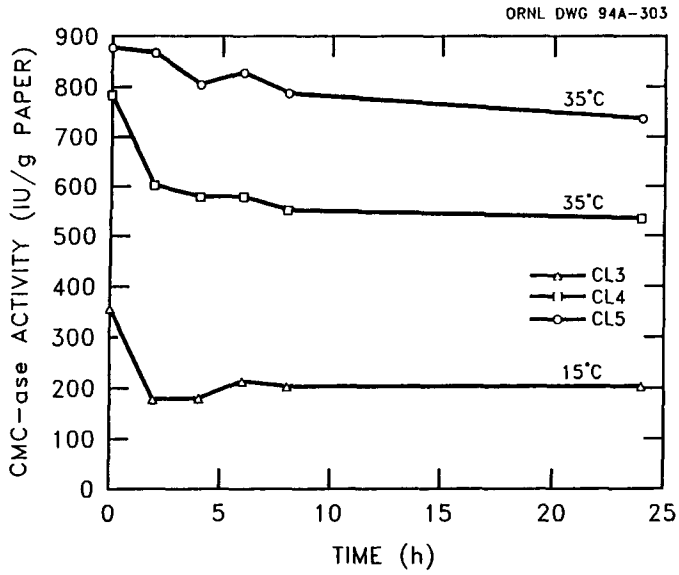

Fig. 2. CMC-ase activity in the reactor liquid as a function of run time.

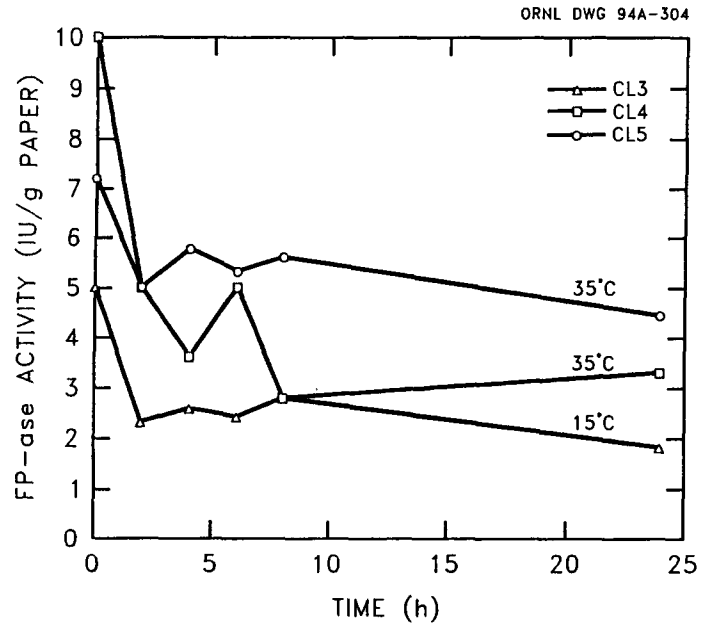

Fig. 3. FP-ase activity in the reactor liquid as a function of time.

as a result of such mechanisms as thermal or shear-induced deactivation. As expected, HPLC analysis of a typical sample indicated that the majority product (95 + %) was cellobiose, because this bacterial source of cellulase does not contain a cellobiase component.

Figure 4 contains a plot of glucose equivalent concentration as a function of time for the three experiments shown in Figs. 2 and 3. The right-

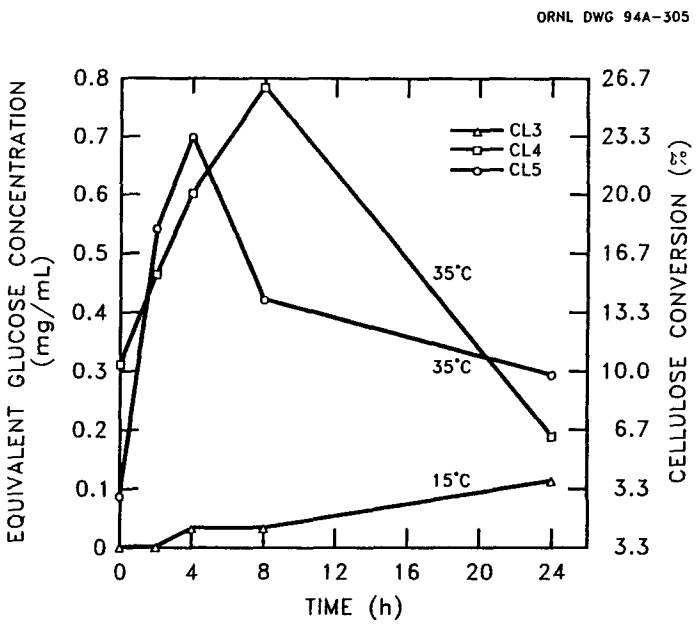

Fig. 4. Equivalent glucose concentration and percent cellulose conversion in the reactor liquid as a function of time. CL3 was carried out at 15°C, whereas CL4 and CL5 were duplicate runs carried out at 35°C.

hand-side *y*-axis contains an approximate percentage conversion of cellulose assuming that the major product is cellobiose. The waste paper was approx 60% cellulose. Initial samples were taken 5 min after introduction of all of the reactor components. It is evident that the hydrolysis reaction does not proceed rapidly at 15°C as evidenced by a 3.7% conversion of cellulose after 24 h of reaction time. This lower temperature did, however, inhibit the growth of contaminant microorganisms in the reactor. Runs CL4 and CL5 show substantial conversion of cellulose at 35°C. In both cases, the reducing sugars concentration rapidly increases in the reactor liquid with equivalent conversions that are around 25% in 4–8 h. Unfortunately, after 4 h two effects decrease the apparent conversion rate and eventually lead to a degree in measured reducing sugars: (1) possible inhibition by cellobiose, and 2) observation of growth of microbial contaminants that consume reducing sugars.

These preliminary experiments have shown that cellulase from NCIB 10462 can be effectively produced and easily recovered for use in waste-paper hydrolysis experiments. In addition, it has been shown that the enzyme is reasonably stable in the temperature range tested over a 24-h period in the attrition reactor system. Optimal pH and temperature conditions have not yet been identified for this enzyme source. The ultimate goal is to find (pH and temperature) conditions under which enzyme activity, conversion, and contaminant growth can be balanced to obtain a viable process. The waste paper used in this study was not autoclaved

because this would represent a type of pretreatment of the cellulose source and is undesirable for the ultimate process application. In future experiments, antibiotics and antifungal agents will be added to inhibit growth of microbial contaminants to study enzyme kinetics, and the paper may be autoclaved to cut down on microbial contaminant effects. HPLC analyses will be used to measure sugar concentrations more precisely, and a more well-characterized waste-paper source will be used to allow direct comparison of this approach to others.

ACKNOWLEDGMENTS

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