0273-2289/97/63–65—0585$10.75

Use of a New Membrane-Reactor Saccharification Assay to Evaluate the Performance of Cellulases Under Simulated SSF Conditions

Effect on Enzyme Quality of Growing *Trichoderma reesei* in the Presence of Targeted Lignocellulosic Substrate

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ABSTRACT

A new saccharification assay has been devised, in which a continuously buffer-swept membrane reactor is used to remove the solubilized saccharification products, thus allowing high extents of substrate conversion without significant inhibitory effects from the buildup of either cellobiose or glucose. This diafiltration saccharification assay (DSA) can, therefore, be used to obtain direct measurements of the performance of combinations of cellulase and substrate under simulated SSF conditions, without the saccharification results being complicated by factors that may influence the subsequent fermentation step. This assay has been used to compare the effectiveness of commercial and special in-house-produced *Trichoderma reesei* cellulase preparations in the saccharification of a standardized microcrystalline (Sigmacell) substrate and a dilute-acid pretreated lignocellulosic substrate. Initial results strongly suggest that enzyme preparations produced in the presence of the targeted lignocellulosic substrate will saccharify that substrate more effectively. These results call into question the widespread use of the "filter paper assay" as a reliable predictor of enzyme performance in the extensive hydrolysis of substrates that are quite different from filter paper in both physical properties and chemical composition.

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Index Entries: Cellulase digestion; *Trichoderma reesei*; pretreated hardwood; diafiltration saccharification; membrane reactor.

INTRODUCTION

Cellulase enzymes are widely sold, and their industrial utilization projected, on the basis of the "filter-paper unit" of activity. The "filter-paper assay" *(1)* is, however, severely limited as a predictor of cellulase performance in the extensive (80–90%-plus) saccharification of actual industrial lignocellulosic substrates. These limitations are traceable both to the chemical and physical differences between filter paper and the industrial substrates, and to the nonhomogeneous nature of most cellulosic substrates (filter paper included). As a result of the nonhomogeneity of the substrates, assays that are run to very limited extents of conversion (such as the 4% conversion target in the filter-paper assay) *(1)* measure the digestibility of only the most easily digestible fraction of the substrate, and reveal little about the convertibility of the bulk of the substrate.

Actual performance of cellulases is estimated better by assays that utilize the actual application substrate *and* are run to the extents of conversion required in the process. Because of the inhibitory nature of the products of cellulase action (primarily glucose and cellobiose), such high-conversion assays encounter the problem of significant product inhibition, if run as simple saccharifications in "closed" systems *(2–5)*. Assays in which product inhibition is a significant factor cannot reliably predict the performance of a cellulase/substrate combination under conditions of simultaneous saccharification and fermentation (SSF), where consumption of the solubilized sugars by the fermentative organism holds the concentrations of saccharification products to very low pseudo-steady-state levels. One obvious strategy for getting around the problem of cellulase inhibition by saccharification products is simply to use actual small-scale SSF as the assay of cellulase effectiveness. This, however, provides a very indirect measure of cellulase performance, because the final results depend not only on cellulase performance, but also on the vagaries of microbial metabolism. Such "SSF assays" cannot, for instance, readily distinguish poor overall performance because of cellulase ineffectiveness from that caused by the toxicity to the fermenting organism of substances that may be found in chemically pretreated biomass. To deal effectively with such problems during process development, one must know which class of problem one is encountering. In addition, the quantitation of volatile products (such as ethanol) under the conditions of such small-scale, improvised SSF assays may present more severe analytical challenges than are posed by sugar determination in liquid samples using standard HPLC methods.

The membrane-diafiltration assay described herein achieves both high extents of saccharification of solid cellulosic substrates and the maintenance of low concentrations of inhibitory soluble products, thus

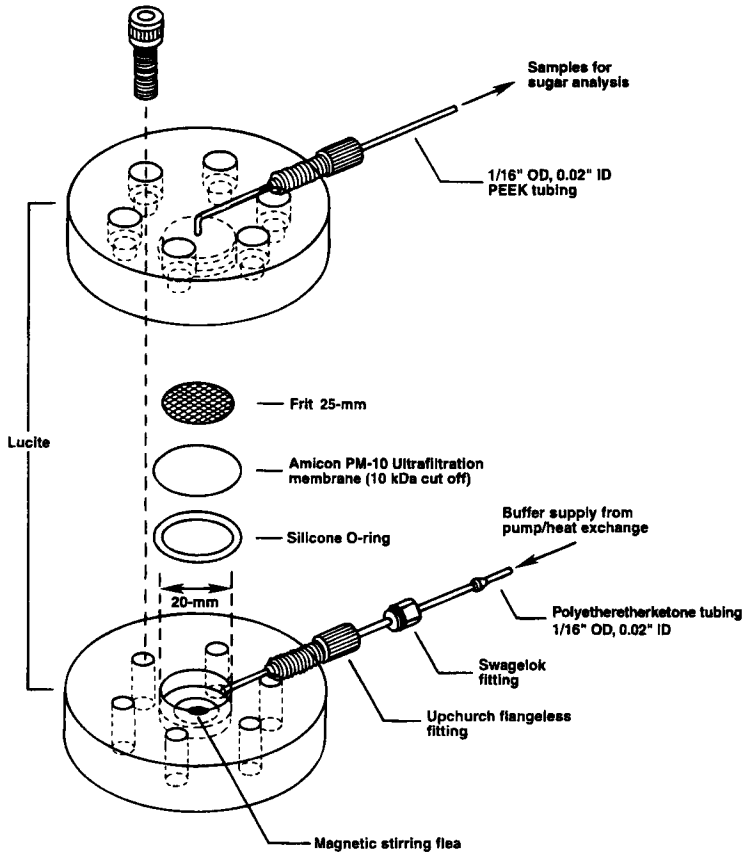

Fig. 1. Schematic representation of the diafiltration saccharification assay (DSA) cell.

effectively mimicking SSF conditions. In the DSA approach, a magnetically-stirred membrane reactor vessel (Fig. 1) is constantly swept by a buffer flux. Cellulase enzymes and solid cellulosic substrate particles are confined to the reaction vessel by an ultrafiltration membrane (10-kDa cutoff) at the exit side of the cell and, at the entrance side of the vessel by the relatively high linear velocity of incoming buffer passing through the small-diameter (0.02-in) entrance port. Soluble saccharification products are swept through the ultrafiltration membrane and out of the cell for detection by HPLC.

Figure 2 presents a schematic diagram of the overall apparatus used in collecting diafiltration-saccharification data. Essential components are an HPLC pump used to deliver buffer, at highly consistent flow rates, through a heat-exchanger to the "high-pressure" side of a custom-built membrane reactor that is temperature-controlled in an oven, and ultimately to a fraction collector set to collect timed fractions. The saccharification progress curve is monitored by weighing the tared fraction-collector tubes (which, for these dilute solutions, gives a satisfactorily accurate estimate of the fraction volume) and then determining the sugar concentra-

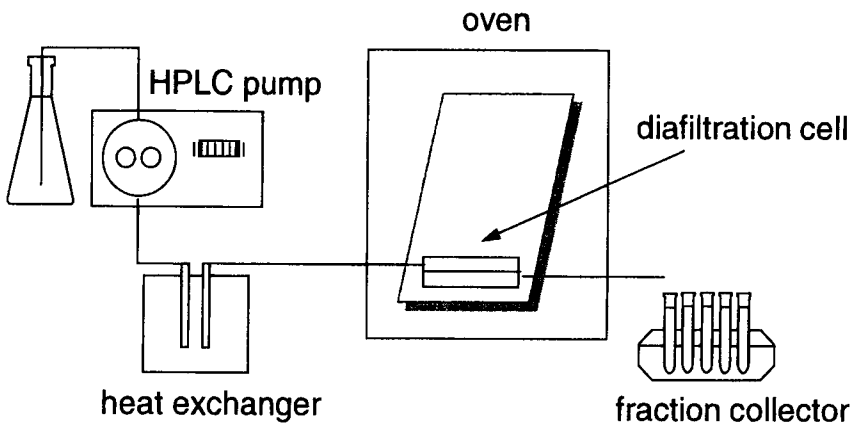

Fig. 2. Schematic of the overall DSA system.

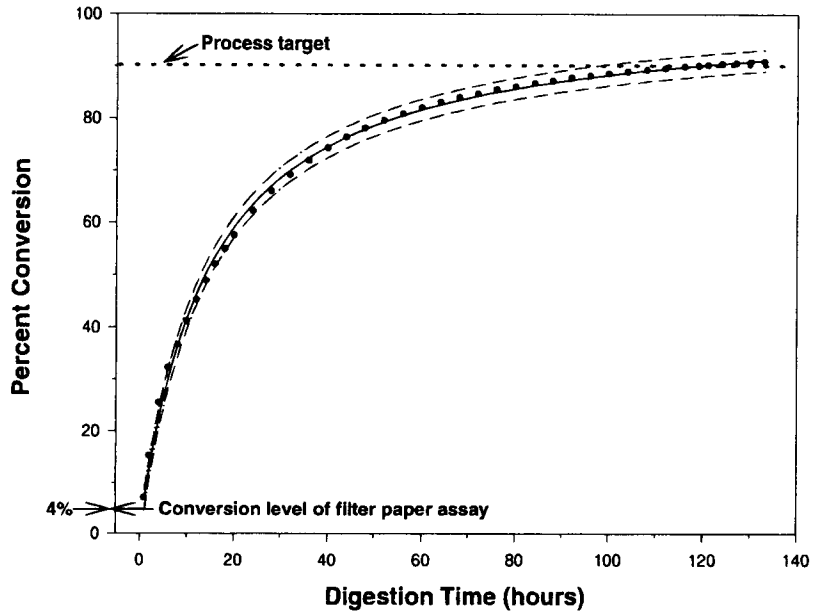

Fig. 3. Illustrative example of DSA data, comparing the relative magnitudes of the cumulative substrate conversion in the DSA with those of the target conversions in the filter-paper assay and in the bioethanol process. Total protein loading (*T. reesei* EG I + CBH I in a 40:60 molar ratio) was equivalent to the protein loading in a 20-FPU/g cellulose loading of native *T. reesei* cellulase. Substrate was 5% (w/v) Sigmacell type 20, in pH 5.0, 20 m*M* acetate; reaction temperature was 50°C. The solid curve is a nonlinear best fit to the data; the dotted lines delineate the 95% confidence interval.

tions of the fractions by HPLC. The cumulative sugar production is then compared with the quantity of cellulose originally loaded into the reactor, as illustrated in Fig. 3, which also graphically compares the extents of conversion achieved in typical diafiltration assays with the target conversion for the filter paper assay.

The basic idea behind the diafiltration assay was presented in 1975 by Howell and Stuck *(6)*, who used a standard membrane concentrator with periodic manual buffer replenishment to accomplish the same result. The advantages of our custom-built apparatus are that

1. It has a substantially smaller volume, which reduces the required quantities of purified enzymes when such are being assayed;
2. It maintains a constant reactor volume and well-controlled buffer flux, thus allowing accurate description of the product concentrations seen by the enzymes at different times during the reaction; and
3. It can operate unattended for days, if need be.

MATERIALS AND METHODS

Diafiltration Assay Apparatus Construction

The diafiltration reactor cell was milled from Lucite to have a "high-pressure" chamber 2.2 mL in volume, and utilizes standard 25-mm diameter Amicon PM-10 membranes (10,000 MW cutoff) and the corresponding Amicon O-rings. Inlet and outlet ports are 11-cm lengths of polyetheretherketone (PEEK) tubing with 0.02-in inside diameter, connected to milled, threaded ports on the cell and connected to input and output lines with Swagelok fittings. The precell heat-exchanger shown in Fig. 2 is formed from a coiled, 10-ft length of 1/8 in. stainless-steel tubing.

Sugar concentrations in collected effluent fractions were determined by chromatography on a Bio-Rad HPX-87P (lead-form) carbohydrate analysis column (Bio-Rad deashing precolumns), installed in a Hewlett-Packard 1090 chromatograph and operated at 85°C with deionized water as mobile phase at a flow rate of 0.6 mL/min.

Enzyme Sources

As an example of commercial cellulase produced using soluble sugar as both inducer and carbon source *(7)*, Iogen Cellulase, lot PRC-191095, was obtained from Iogen, Inc., Ottawa, Ontario, Canada. Spezyme CP, lot GC 30952.3E1P1Z1, was obtained from Genencor, South San Francisco, CA. Substrate-induced *T. reesei* cellulase (hereinafter referred to as "PYP-grown cellulase") was produced in our laboratory by growing the organism (NREL strain MTCA-13, which was derived from MG-80) in the presence of 1% pretreated yellow poplar and 6% lactose.

The growth medium used in this study was based on the original Mandels medium *(8)*, substituting corn steep with a 50/50 mixture of peptone and yeast extract. The exact composition was: $CaCl_2$, 0.4 g/L; $MgSO_4$, 0.3 g/L; KH_2PO_4, 2 g/L; $(NH_4)_2SO_4$, 1.4 g/L; peptone, 5 g/L; yeast extract, 5 g/L; Tween 80, 0.2 mL/L; and trace mineral solution.

All the fermentations were carried out using 10 L of the above medium in New Brunswick Scientific (NBS) 14 L Microferm bioreactors (series MGF114) with agitation and temperature control. The pH was controlled using a model 7600 Cole-Parmer controller with an Ingold probe. Dissolved oxygen level was maintained using an NBS dissolved oxygen controller model DO-81 and an Ingold galvanic probe. The fermentation vessel was modified by adding a port to allow for homogenous sampling from the bottom of the reactor.

The media components described previously *(9)* were sterilized in the presence of the pretreated poplar substrate in the bioreactor. Lactose was added aseptically post sterilization. The reactors were allowed to equilibrate to temperature (26°C), at which time the dissolved oxygen and pH probes were calibrated. Adjustments to pH were automatically controlled with the additions of 3*M* H_3PO_4 and 3*M* NH_4OH. The concentration of dissolved oxygen was maintained at 20% by the automatic adjustment to the flow rate of a 50:50 mixture of compressed air and pure oxygen. The fermentation mixture was constantly mixed at 300 rpm for maximum oxygen mass transfer and minimum shear.

The fermentations were inoculated with a 10% culture produced in two shake flasks; this constituted time zero. Fifty-mL samples were taken at 24-h intervals until the cellulase production leveled. The activity was determined using the FPU assay *(1)* or general glucose release from filter paper and recorded as a function of fermentation time.

Cellulosic Substrates

Microcrystalline cellulose (Sigmacell, Type 20) was purchased from Sigma Chemical (St. Louis, MO), and was weighed, dry, into the cell. Pretreated yellow poplar (PYP) sawdust used in SSF studies was subjected to dilute-acid pretreatment as described previously *(9)*. PYP used in DSAs was further subjected to wet milling using an Ultra-Turrax (Tekmar, Cincinnati, OH) to reduce the particle size to a distribution in the same general range as that of Sigmacell, type 20. This finely divided material was then exchanged into 20 m*M* acetate buffer, pH 5.0, containing 0.02% (w/v) sodium azide. A procedure was developed for pipetting from a well-stirred 3.54% (w/v) slurry of this material to produce a series of standardized substrate aliquots that contained an average of 0.0520 g (dry weight) of PYP, with a standard deviation (n = 4) of ±0.7%. Two of these aliquots were quantitatively transferred to the diafiltration cell as PYP loadings for each assay.

Compositional analysis, carried out as described in reference 9, revealed that the PYP consisted of 67.7% glucan, 1.68% xylan, 32.42% Kjehldal lignin, 1.45% acid-soluble lignin, and 0.39% total ash. Particle-size analysis of both substrates was carried out using a Coulter LS-130 light-scattering analyzer with a fluid module.

Simultaneous Saccharification and Fermentation

Effectiveness of the two enzyme preparations was compared following the standard (shake-flask, quadruplicate) procedure described earlier *(9)*, except that the enzyme loadings were different from those used in the previous study.

Diafiltration Assay Conditions

All DSAs were carried out at pH 5.0 in 20 m*M* sodium acetate buffer containing 0.02% (w/v) sodium azide to prevent microbial growth, at either 37 or 50°C. All substrate loadings were equal in terms of cellulose content, being set at 70.4 mg cellulose in each 2.2-mL digestion mixture. In the case of PYP, a loading of 104 mg (dry wt) of biomass was required to achieve this cellulose loading. Solids loadings (as opposed to cellulose loadings) were thus approx 3.2% (w/v) for the microcrystalline cellulose, and approx 4.7% for PYP. Cellulase enzyme loadings were based on cellulose content of the substrate and were fixed at 20 FPU/g cellulose, using filter-paper activities determined in our laboratory for both enzyme preparations. Buffer flux through the 2.2-mL reaction cell was 0.072 mL/min.

RESULTS AND DISCUSSION

Substrate Particle-Size Analysis

Both substrates used in the DSA cell were finely divided material. As shown in Fig. 4, the two size-distributions are significantly different, but the two substrates, Sigmacell Type 20 and PYP, can be described as roughly comparable in terms of particle size. If simple particle size should affect access by the enzymes to potential cleavage sites, the "smaller" Sigmacell substrate would be favored.

SSF Comparisons

Figure 5 shows the performance of the two enzyme preparations in the SSF of PYP. At both the 5 and 10 FPU/g enzyme loadings, the SSF mixtures containing the enzyme produced by *T. reesei* in the presence of PYP outperformed the commercial preparation grown on soluble sugars alone, although the preparations were used at equal loadings in terms of "filter-paper activity." This is not an especially surprising result, the suggestion having been made earlier that the presence of the actual intended "target" biomass during enzyme production may induce the production of more appropriate ratios of major cellulase components, or of minor but important ancillary enzymes important for digesting complex substrates *(10–12)*. We believe, however, that until now there has been little if any published systematic experimental testing of this idea.

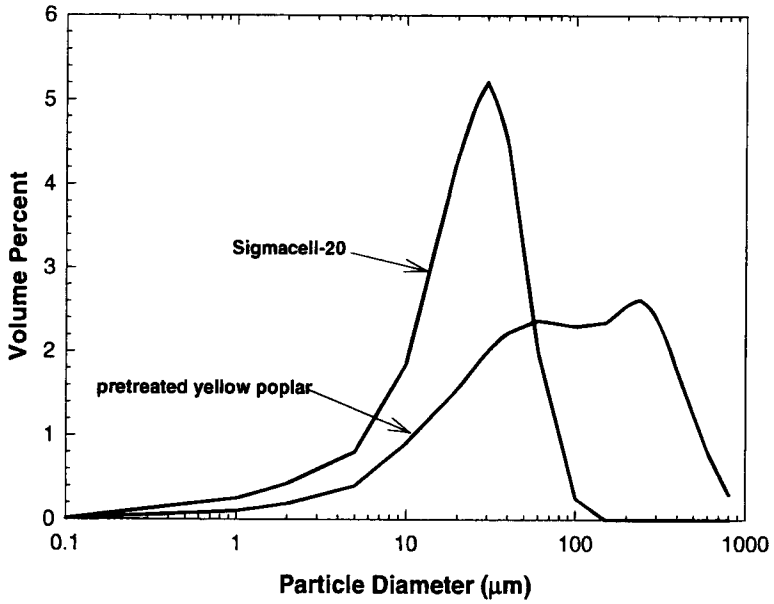

Fig. 4. Comparative particle size distribution for the two insoluble cellulosic substrates used in this study, Sigmacell type 20 and pretreated yellow poplar (PYP).

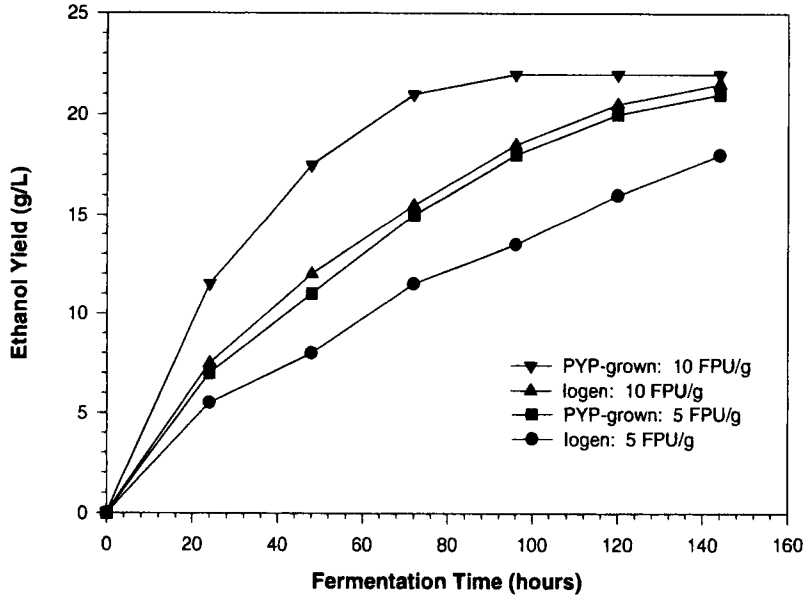

Fig. 5. Use of actual SSF assay to evaluate the relative effectiveness of PYP-grown *T. reesei* cellulase and commercial cellulase grown on soluble sugars alone, in the fermentation of PYP. Fermentations by *S. cerevisiae* D_5A were carried out at 37°C at pH 5.0 in a total volume of 100 mL in 250-mL Erlenmeyer flasks shaken at 15/s. Solids loading was 10%, with enzyme loadings of either 5 FPU or 10 FPU of the tested cellulase per g cellulose, plus 25 βGU (NOVO SP188) per g cellulose.

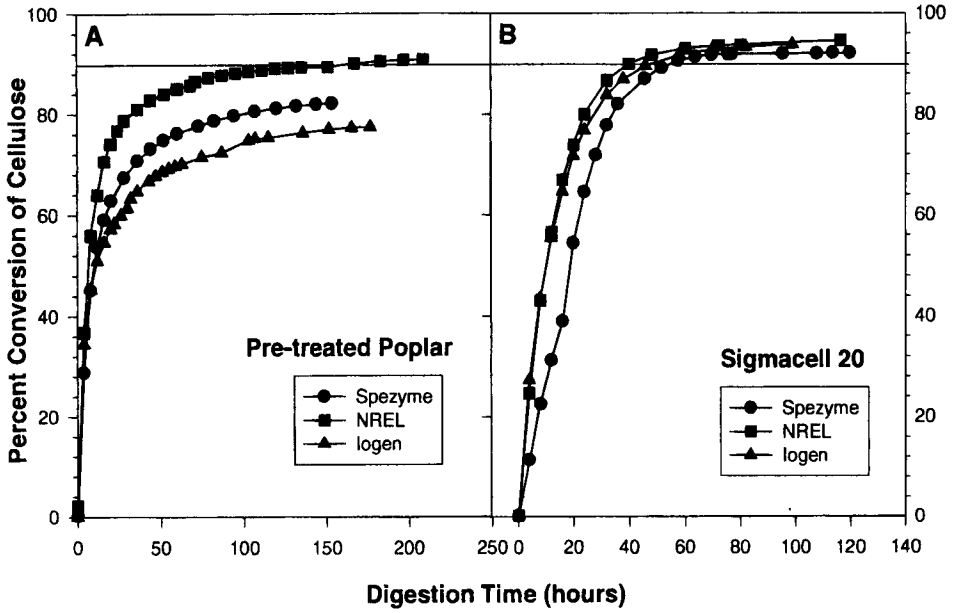

Fig. 6. Use of the direct DSA to evaluate the effectiveness of three different *T. reesei* enzyme preparations in saccharifying **(A)** PYP and **(B)** microcrystalline cellulose (Sigmacell, type 20) at pH 5.0 and 37°C. Substrate concentrations were 70.4 mg cellulose in each 2.2-mL reactor volume; in the case of PYP, this cellulose loading was achieved by loading 104 mg of pretreated biomass in each cell. Enzyme loadings were fixed at 20 FPU/g cellulose, based on filter-paper activities determined in our laboratory.

Diafiltration-Saccharification Comparisons

In addition to being both interesting and significant in their own right, the SSF results provided an opportunity to "calibrate" the DSA as a method of predicting SSF performance by combinations of cellulase enzymes and cellulosic substrates. As shown in Fig. 6A, the directly-measured saccharification results correlate well with the SSF results, in that the enzyme produced in the presence of PYP is markedly superior, both in terms of kinetics and apparent ultimate yields, in the saccharification of PYP. The PYP-grown enzyme reaches 80% conversion in less than 33 h digestion; at this time a equivalent loading (in terms of filter-paper activity) of the commercial preparation, produced on soluble sugars alone, has reached only 63% conversion, and does not in fact reach the 80% level even by 176 h. The PYP-grown enzyme goes on to achieve 90% conversion of the cellulose content by 182 h. The performance of a second commercial cellulase preparation, Spezyme (Genencor International) is intermediate between the performances of the NREL and Iogen enzymes.

The saccharification progress curves shown in Fig. 6B strongly suggest that the differences seen in Fig. 6A are substrate-dependent. In the digestion of microcrystalline cellulose (Sigmacell, type 20), equivalent filter-

paper-activity loadings of the two enzymes perform equivalently (Fig. 6B). The major compositional difference between the two substrates is that Sigmacell is essentially all cellulose, whereas PYP (as noted under MATERIALS AND METHODS) is more than 32% Kjehldal lignin. It may be that the breaking of tight lignin carbohydrate complexes is key to the enhanced kinetics and 11%+ better ultimate yield (relative to that of the Iogen preparation) shown by the PYP-grown enzyme in the digestion of the PYP as a substrate. If so, either breakdown products from the substrate, or the simple presence of the macromolecular matrix during production of the enzyme mixture, may serve as an inducer of an enzyme (or enzymes) that may be minor in quantity of protein, but very important in terms of activity on actual process substrates.

CONCLUSIONS

The ability to obtain 80–90% conversion of biomass cellulose by SSF in 5–7 d at reasonable cellulase enzyme cost is considered to be an important requirement for economic viability of an SSF-based bioethanol process *(13,14)*.

The results of this study strongly suggest that production of cellulase enzyme mixtures in the presence of the actual target substrate is a useful approach to optimizing enzyme mixtures for reaction time and yield criteria. Furthermore, it has been demonstrated that the DSA assay is an effective and enzyme-efficient means of predicting the SSF performance of enzymes according to both of these criteria.

ACKNOWLEDGMENTS

This work was funded by the Biochemical Conversion Element within the Biofuels Systems Program of the Office of Fuels Development of the US Department of Energy.

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