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Analysis of Biomass Cellulose in Simultaneous Saccharification and Fermentation Processes[1]

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ABSTRACT

A direct method for determining the cellulose content of biomass residues resulting from simultaneous saccharifiaction and fermentation (SSF) experiment has been developed and evaluated. The method improves on classical cellulose assays by incorporating the enzymatic removal of yeast glucans from the biomass residue prior to acid hydrolysis and subsequent quantification of cellulose-derived glucose. An appropriate cellulase-free, commercially available, yeast-lysing enzyme preparation from *Cytophaga* was identified. A freeze-drying step was identified as necessary to render the SSF yeast cells susceptible to enzymatic lysis. The method was applied to the analysis of cellulose and yeast-associated glucans in SSF residues from three pretreated feedstocks; hybrid poplar, switchgrass, and cornstover. Cellulose assays employing the lysing-enzyme preparation demonstrated relative errors up to 7.2% when yeast-associated glucans were not removed prior to analysis of SSF residues. Enzymatic lysis of SSF yeast cells may be viewed as a general preparatory procedure to be used prior to subsequent chemical and physical analysis of SSF residues.

Index Entries: Saccharification; fermentation; yeast; cellulose; lysing enzyme.

INTRODUCTION

Simultaneous saccharification and fermentation (SSF) is a process involving the simultaneous enzymatic saccharification of biomass cellu-

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lose and the microbial fermentation of the resulting glucose to ethanol. It has been reported that SSF is superior to the analogous two-stage process, enzymatic saccharification followed by yeast fermentation, for the following reasons: lower enzyme loads may be used, because saccharification products that may inhibit cellulases, particularly glucose and cellobiose, are removed from the reaction mixture via yeast fermentation; equipment costs are reduced owing to the use of a single reactor; and contamination is less of a problem owing to the relatively low pH (~5) and ethanol content of reaction mixtures *(1–3)*.

Biomass conversion efficiency in the SSF process is generally evaluated in terms of the percentage of total cellulose converted to ethanol and/or the absolute amount of ethanol produced. Values based on the percent of total cellulose converted allow direct comparison of saccharification efficiencies between those processes which do not include fermentation. Generally, the amount of cellulose consumed in an SSF process is estimated by a summative analysis of the major products resulting from cellulose degradation *(4–8)*. The major saccharification products are glucose and cellobiose; the major fermentation product is ethanol. The summative approach requires one to assume that an unknown amount of saccharification product is used for yeast cell growth. Saccharification product that goes into cell growth is obviously not measured as either glucose, cellobiose, or ethanol. It must generally be assumed that 5–10% of the total saccharification product is incorporated into new cell growth, because the actual amount going to cell growth is very hard to quantify *(5,9)*. The summative approach suffers from the inaccuracy associated with this assumption. Further limitations of the summative approach are that the final value obtained for total cellulose consumed will reflect the additive error from each of the separate product analyses, that minor products are not directly assayed, and that it is labor intensive.

An alternative approach for estimating the amount of cellulose consumed in an SSF process is to measure directly the amount of residual cellulose remaining in the SSF system at the completion of the experiment. The measured amount of residual cellulose at time "*t*" is then subtracted from the amount of cellulose at time "0" to obtain the amount of cellulose that has been consumed in the SSF process through time "*t*". The approach is straightforward and it avoids many of the problems associated with the summative approach. However, the analytical techniques necessary for the implementation of this more direct approach have not been satisfactorily developed. To measure the cellulose content of an SSF residue one typically hydrolyzes the cellulose-containing residue with aqueous sulphuric acid and then quantitatively measures the glucose present in the hydrolysate. It is assumed that all of the glucose found in the hydrolysate resulted from the hydrolysis of cellulose. This assumption presents a problem for SSF systems, because in some

situations a significant portion of the glucose that is generated during the hydrolysis step may arise from the yeast cells associated with the SSF residue. Yeast cell walls contain a beta-1,3-linked glucan polymer that is hydrolyzed under conditions used to hydrolyse cellulose *(10,11)*. The presence of appreciable quantities of yeast-derived glucose will obviously result in an overestimate of cellulose associated with the SSF residue.

Two different approaches have been used to correct for the problem of yeast-associated glucans in SSF residues. In one approach, the yeast glucans are prehydrolyzed with 3% HCl and separated (washed) from the insoluble residue prior to the measurement of cellulose *(5,9,12)*. This approach is based on the observation that yeast glucans are hydrolyzed under less stringent conditions than those required for the complete hydrolysis of cellulose. However, this method tends to underestimate the cellulose content of SSF residues because the process used to hydrolyse the yeast cell-wall polymers may also hydrolyse the more susceptible fractions of the biomass cellulose. Wyman et al. *(12)* indicated that roughly 5% of the cellulose component of biomass is susceptible to hydrolysis under the conditions employed for the acid-hydrolysis of yeast-cell walls. A second approach is to hydrolyze exhaustively the residual cellulose with cellulase enzymes in the absence of fermenting yeasts *(1)*. At a specified time, the active yeasts in an SSF mixture are poisoned with NaF, excess cellulase enzyme is then added, and the mixture is incubated until no further glucose is generated. The amount of glucose generated following the poisoning of the yeast cells is taken as proportional to the amount of residual cellulose in the SSF residue. This method avoids the hydrolysis of yeast glucans, provided that the cellulase enzyme preparations employed for cellulose saccharification do not contain hydrolytic enzymes active toward yeast glucans. This method suffers from another limitation, however, in that it is likely that a fraction of the total cellulose component of a biomass sample will not be accessible for enzymatic digestion. Hence, the application of this method to SSF residues is likely to underestimate the residue's actual cellulose content. The degree of underestimation is likely to be a function of the severity of the pretreatment the biomass has undergone prior to the SSF process—because this is known to affect extents of cellulose saccharification *(13,14)*.

Here we present an alternative direct approach for measuring the cellulose content of SSF residues. The method is based on the hydrolysis of yeast cell-wall glucans with commercially available cellulase-free hydrolytic enzymes, then separation of the hydrolyzed-yeast glucans from the insoluble SSF residue and subsequent quantitative analysis of the cellulose-containing SSF residue. This approach to cellulose analysis circumvents the limitations associated with the direct approaches discussed above.

MATERIALS AND METHODS

Hybrid poplar, switchgrass, and a cornstover mixture were obtained from the National Renewable Energy Laboratory, Golden, CO. A *Trichoderma reesei* cellulase preparation (59 FPU/mL, 57 beta-glucosidase units/mL—units defined as described by Ghose, ref. *15*) was obtained from Environmental Biotechnologies, Inc., CA. *Saccharomyces cerevisiae* (D_5A), genetically derived from Red Star Brewers yeast (1) and provided by the National Renewable Energy Laboratory, was used as the fermentation yeast. Yeast extract-peptone-dextrose (YPD) media was 1% yeast extract (Difco), 2% peptone (Difco), and 2% glucose, adjusted to pH 5.0. Lysing enzyme (from *Cytophaga* species, product #L9893), α-cellulose and the glucose analysis reagents (glucose oxidase/peroxidase) were purchased from Sigma Chemical, St. Louis, MO. The lysing enzyme preparation had 164 U/mg solid (656 U/mg protein)—a unit being defined as the glucanase, protease, and cell-lytic activity on Brewers yeast in 50 mM phosphate buffer, pH 7.0, at 37°C (1U is equivalent to that amount of enzyme that causes a 1% decrease in OD at 670 nm in 1 min).

Dilute Acid-Pretreatment of Biomass Substrates

Dilute acid-pretreatment of biomass substrates was done in batch reactors as described by Fenske *(16)*. Pretreatment conditions for the switchgrass, poplar, and cornstover substrates were 1.2% H_2SO_4/180°C/0.5 min., 1% H_2SO_4/180°C/0.56 min, and 1.2% H_2SO_4/180°C/0.9 min, respectively. Following pretreatments, residual biomass was washed with distilled water until the filtrate was neutral. Washed solids were stored at 4°C until used in experiments.

Enzymatic Lysing of Yeast-Cell Walls

Yeast cells used as substrate for the lysing assays were prepared by transferring a frozen stock culture (1 mL) into 50-mL YPD media for 12 h and then transferring to 400-mL YPD and incubating at 38°C for 4 d. Cells were then harvested by centrifugation (1500g, 10 min), washed with distilled water and freeze-dried. For standard assays, substrate (yeast cells, 30 mg dry cells; α-cellulose, 300 mg; Whatman #1 filter paper, 250 mg; pretreated switchgrass, 300 mg) and enzyme were incubated in 10 mL of 50 m*M* sodium phosphate buffer, pH 7.0, at 37°C with orbital agitation. Enzyme loads varied as indicated in the text. The influence of biomass on lysing-enzyme activity was determined by supplementing standard assay solutions containing 0.5 mg/mL lysing enzyme with biomass (pretreated or native) to 3%. The amount of glucan associated with a given quantity of yeast cells was determined by summing the total glucose equivalents resulting from the complete acid hydrolysis of all yeast glycans.

Cycloheximide (0.1%) and tetracycline (0.1%) were added to all reaction mixtures to prevent yeast growth and bacterial contamination, respectively. Following predetermined incubation times, yeast-glucan hydrolysis was followed by measurement of soluble glucose. Disappearance of intact yeast cells was monitored microscopically at 400 to 1000X.

Simultaneous Saccharification and Fermentation

SSF conditions were essentially those outlined by Philippidis et al. *(17)*. SSF was done in 250-mL Erlenmeyer flasks equipped with water traps, initiating the experiment with a 2:5 medium:flask volume ratio. Flasks were incubated at 38°C with orbital agitation (150 rpm). On initiation of the SSF experiment a typical sample contained 3% cellulose, 25 FPU/g cellulose of cellulase activity, 10% (v/v) yeast inoculum (log phase cells), in YPD medium to give a total liquid volume of 100 mL. SSF incubations were terminated after 7 d by immersion of the reaction flask in boiling water for 10 min. The SSF insoluble residue was then separated from the soluble phase by centrifugation and decanting, and then washed repeatedly with distilled water. The washed SSF residue was freeze-dried and stored desiccated until analyzed.

Cellulose in SSF Residue

The freeze-dried SSF residue was assayed for total cellulose by two methods. In the first method, a 300-mg sample was incubated at 30°C in 3 mL of 72% sulphuric acid for 2 h. The sample was then diluted to 4% sulphuric acid with distilled water and autoclaved for 1 h. The resulting hydrolysate was assayed for glucose using a coupled glucose oxidase/peroxidase assay. The total glucose content of the hydrolysate was used to calculate the cellulose content of the SSF residue. This approach is widely used for the quantification of biomass cellulose *(16,18,19)*. The second method mimics the first with the exception that the SSF residue is treated with lysing enzyme for the removal of yeast glucans prior to the acid-hydrolysis step. Yeast cell glucans were solubilized by incubating 300 mg SSF residue in 10 mL lysing buffer (see above) containing 0.5 mg enzyme per mL for 3 d at 38°C. Solubilized glucans were separated from SSF biomass residue by centrifugation (1500*g*, 10 min) and decanting, and repeated washing with distilled water.

RESULTS AND DISCUSSION

Lysing-enzyme preparations are a complex mixture of enzyme activities, including chitinases, proteases, deoxyribonucleases, and the β-1,3-glucanases responsible for hydrolyzing cell-wall glucans *(20)*. These preparations may also posses significant cellulase (β-1,4-glucanase) activ-

ity (21). To be an effective enzyme preparation for the removal yeast glucans from biomass cellulose, it is essential that the enzyme preparation display minimal cellulase activity. The hydrolytic activity of the Sigma *Cytophaga* lysing enzyme preparation on yeast cells, α-cellulose, filter paper, and pretreated switchgrass is shown in Fig. 1. The results are reported in terms of the amount of soluble glucose generated from each substrate. The enzyme preparation's lack of activity on the three cellulose substrates and its demonstrated ability to hydrolyze yeast glucans make it appropriate for selective removal of yeast glucans from biomass cellulose. This result may not apply to lysing-enzyme preparations obtained from other species of the genus *Cytophagia* since some species can readily degrade cellulose (22). The Sigma preparation was capable of hydrolysing essentially all (~97%) of the theoretically available glucan present in the yeast cells. The time-course of yeast-glucan hydrolysis is shown to be dependent on enzyme load, as expected. The data of Fig. 1 is based on measured glucose, which is the end-product for the complete hydrolysis of these glucans. However, complete hydrolysis is not required for separation of yeast glucans from insoluble biomass. Separation of yeast glucans from particulate biomass will occur when the yeast glucans, or their microenvironment, have been sufficiently modified to insure that they no longer pellet with the biomass residue during the centrifugation phase of the washing process (in this study that corresponds to centrifugation at 1500*g* for 10 min). Thus, the 72 h timepoint indicating complete conversion of yeast glucans to glucose represents an enzyme treatment appreciably more extensive than that required for separation of yeast glucans from biomass cellulose. In this regard, microscopic examination (at 400X) of reaction mixtures corresponding to curve 1 of Fig. 1 showed that most of the yeast cells had been lysed after only 0.5 h reaction time, and that essentially all yeast cells had been lysed by 2 h.

The apparent hydrolytic activity of the *Cytophaga* lysing-enzyme preparation decreased when authentic biomass substrates were included in reaction mixtures. This result is not likely owing to the presence of traditional soluble inhibitors, because the biomass substrates had been washed extensively. A more likely cause is the nonspecific absorption of lysing enzyme to the biomass substrate (23–25). The effect of the presence of a lignocellulosic material on lysing-enzyme activity is in part determined by the history of that particular material. For example, Fig. 2 shows that lysing-enzyme activity was more sensitive to the presence of pretreated switchgrass than to the original native switchgrass. The extent of this apparent inhibition is not large (Fig. 2); in each of the cases depicted in Fig. 2 greater than 80% of the theoretical yeast glucan had been converted to glucose at 48 h of incubation. The higher rates of glucan hydrolysis reported in Fig. 2, compared to those in Fig. 1, are owing to the use of higher enzyme loads in the Fig. 2 experiments. The different rates of glucan

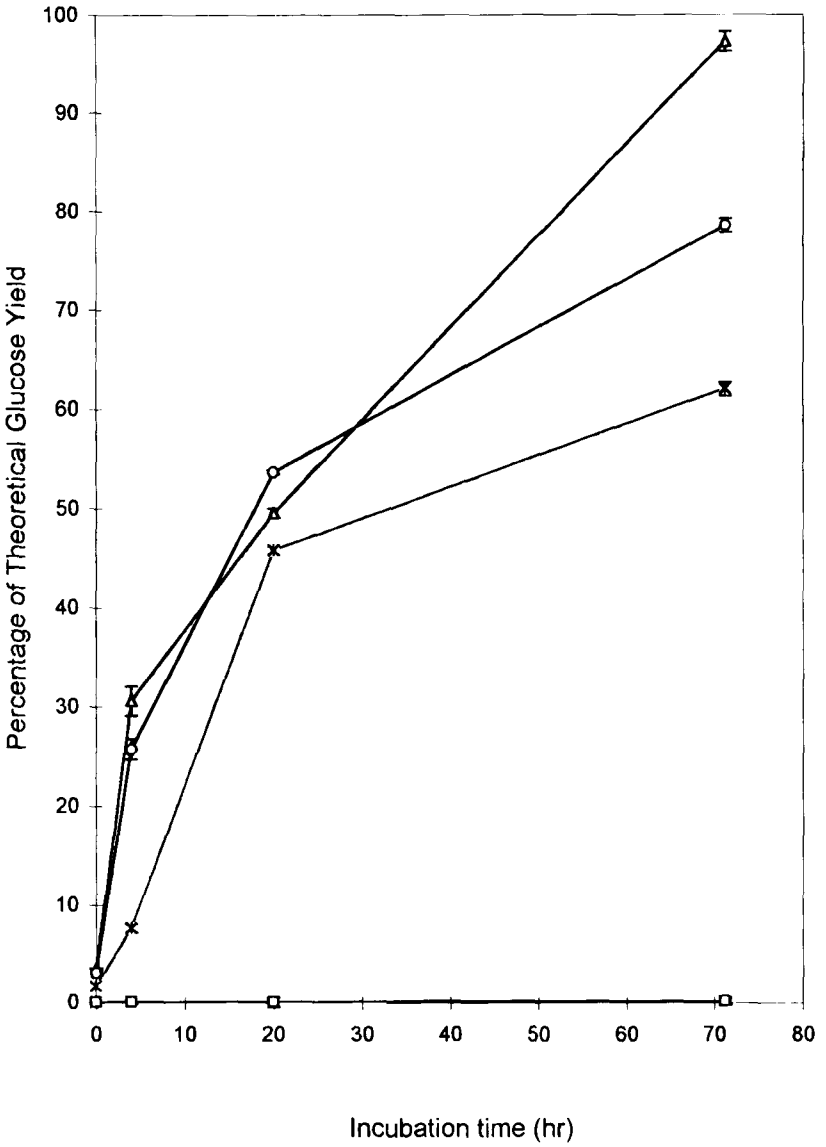

Fig. 1. Time-course for theoretical glucose yield from selected glucan-containing substrates. Treatments were 0.3 wt% yeast cell solids with 0.0125 mg/mL lysing enzyme (*); 0.3 wt% yeast cell solids with 0.124 mg/mL lysing enzyme (○); 0.3 wt% yeast-cell solids with 0.245 mg/mL lysing enzyme (Δ); 2.5 wt% filter paper with 0.124 mg/mL lysing enzyme (□); 2.5 wt% filter paper with 0.5 mg/mL lysing enzyme (●); 3.0 wt% microcrystalline cellulose with 0.124 mg/mL enzyme (■) and 3.0 wt% pre-treated switchgrass with 0.5 mg/mL lysing enzyme (◇). Ten mL reaction mixtures were 50 m*M* sodium phosphate, pH 7.0, at 38°C. Note: the filter paper, microcrystalline cellulose and biomass curves lay on top of each other; hydrolytic products were not detected from these cellulose-based substrates.

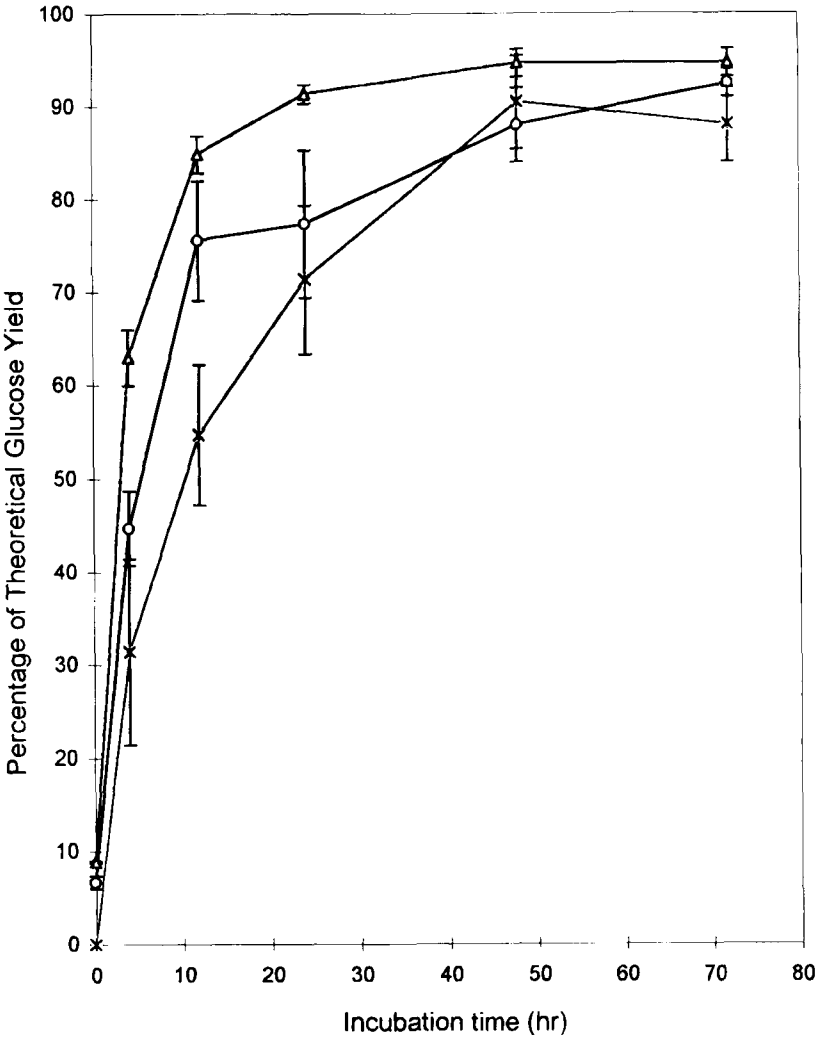

Fig. 2. Time course for theoretical glucose yield from yeast cells incubated with lysing enzyme in the absence of biomass (Δ), in the presence of native switchgrass (○) and in the presence of pretreated switchgrass (✶). Reaction mixtures contained 0.3 wt% yeast-cell solids, 0.5 mg/mL lysing enzyme and, where applicable, 3.0% biomass. Ten mL reaction mixtures were 50 mM sodium phosphate, pH 7, at 38°C. Data are presented as the mean and standard deviation (error bars) of two assays.

hydrolysis in reaction mixtures containing native vs pretreated switchgrass illustrates that the minimum time required for incubation of lysing enzyme with an SSF residue, for purposes of separating yeast glucans from biomass cellulose, will be dependent on the identity and the history of the lignocellulosic substrate. The rate of cell lysis can be effectively increased for all substrates by the using higher enzyme loads.

An effective procedure for determining the incubation time required for efficient separation of yeast glucans from biomass cellulose is as

follows. A predetermined amount of yeast cells, containing a known amount of glucose equivalents, is added to a series of flasks containing the test substrate. A chosen amount of lysing enzyme is then added and the samples are allowed to incubate under optimum conditions. Following appropriate incubation times, representative test samples are centrifuged as in an actual experiment to separate the soluble/suspended yeast glucans from biomass cellulose. The supernatant is then assayed for total glucose equivalents. This assay requires that soluble oligomers be hydrolyzed to monomeric glucose. The time-course for recovery of the yeast glucans in the aqueous phase is thus obtained. A representative plot of such an experiment is shown in Fig. 3 for a pretreated switchgrass substrate. It is clear that under the conditions used for this assay essentially all of the yeast glucan could be separated from the biomass cellulose following 10 h of incubation.

The data for Fig. 2 and the photomicrographs in Fig. 4 illustrate that in model systems the yeast cells were readily lysed and the inherent glucans readily hydrolyzed when incubated with lysing enzyme in the presence of biomass substrates. It was therefore interesting to find that yeast cells which had actually been part of an SSF experiment were resistant to lysing enzyme if the duration of the SSF experiment exceeded 3 d (conclusion based on microscopic observation of intact yeast cells). The resistance to lysing enzyme was observed even at enzyme loads of up to 3 mg/mL. Several modifications of the lysing reaction conditions were tested in an attempt to increase the susceptibility of SSF yeast cells to lysing enzyme. Addition of bovine serum albumin and β-lactoglobulin to decrease nonspecific adsorption of enzyme, addition of the reducing agents β-mercaptoethanol and dithiothreitol to increase yeast cell-wall accessibility *(26)*, and oven-drying prior to enzyme treatment all failed to increase the susceptibility of yeast cells to lysing enzyme. However, it was found that freeze-drying the SSF residue prior to enzyme treatment rendered the yeast cells susceptible to lysing enzyme. No yeast cells could be detected in freeze-dried SSF residues following 24 h incubation with lysing enzyme. The enzyme treatment was found to effectively degrade yeast cells in native and pretreated switchgrass, poplar, and cornstover freeze-dried SSF residues. This technique appears to be applicable to a wide variety of biomass feedstocks and, thus, we recommend that SSF residues be freeze-dried prior to treatment with lysing enzyme.

The molecular basis for the increased susceptibility of freeze-dried yeast cells to lysing enzyme is not clear. It has been reported that freeze-thawing processes may alter the membrane structure of yeast cells and cell outlines *(27–29)*; however, our research did not find changes in cell-wall structure. It is known that intact yeast cells (without prior freeze drying) become more resistant to protoplasting enzymes when they are grown in the stationary phase, and that their susceptibility to these enzymes can be increased by the addition

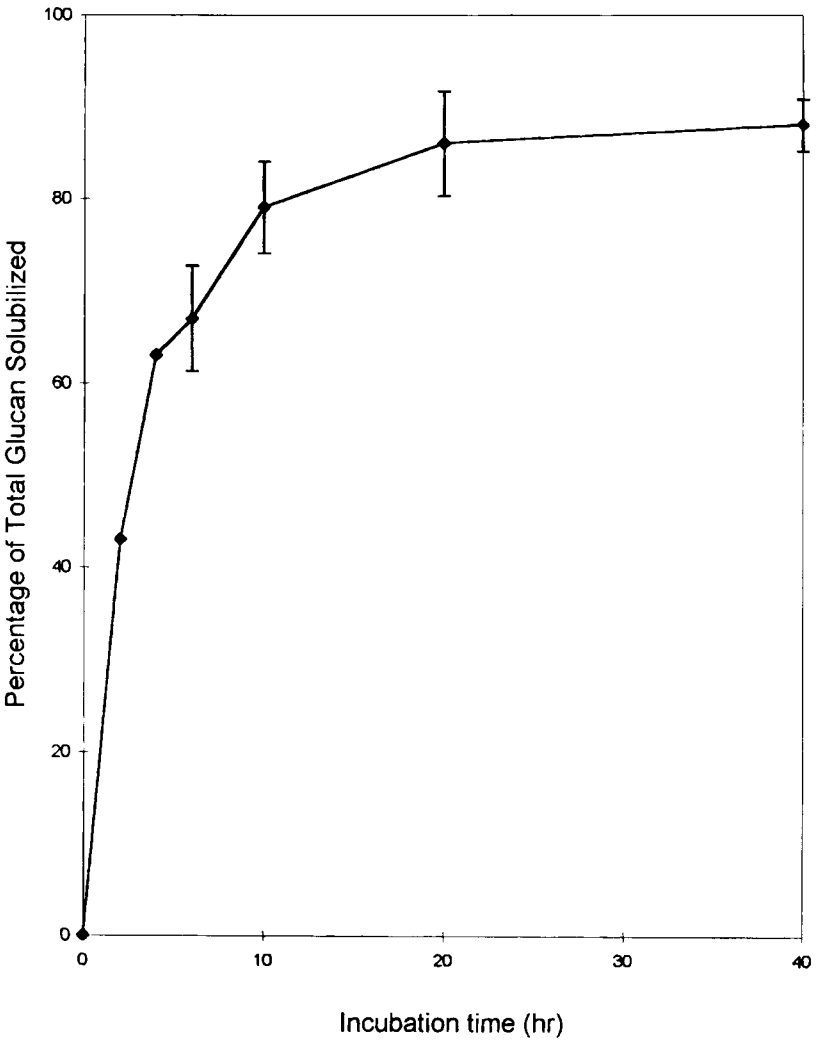

Fig. 3. Time-course for glucan dissolution from yeast cells in the presence of pretreated switchgrass. Reaction mixtures contained 0.3 wt% yeast cell solids, 0.5 mg/mL lysing enzyme, 3.0 wt% biomass in 10 mL reaction mixtures that were 50 m*M* sodium phosphate, pH 7.0, at 38°C. Data are presented as the mean and standard deviation (error bars) of two assays.

of reducing agents *(26,30)*. In this study, the yeast cells became resistant to lysing enzyme when ethanol yields had reached 70–75% of maximum (equivalent to 3 d incubation, Fig. 5). We found that reducing agents did not improve enzymatic lysis of these SSF cells unless the SSF residue was previously freeze-dried. Once the residue was freeze-dried, addition of dithiothreitol to the lysing reaction mixture improved the efficiency of cell lysis.

The application of lysing enzyme to the analysis of the cellulose content of SSF residues from pretreated poplar, switchgrass, and corn stover (stalks

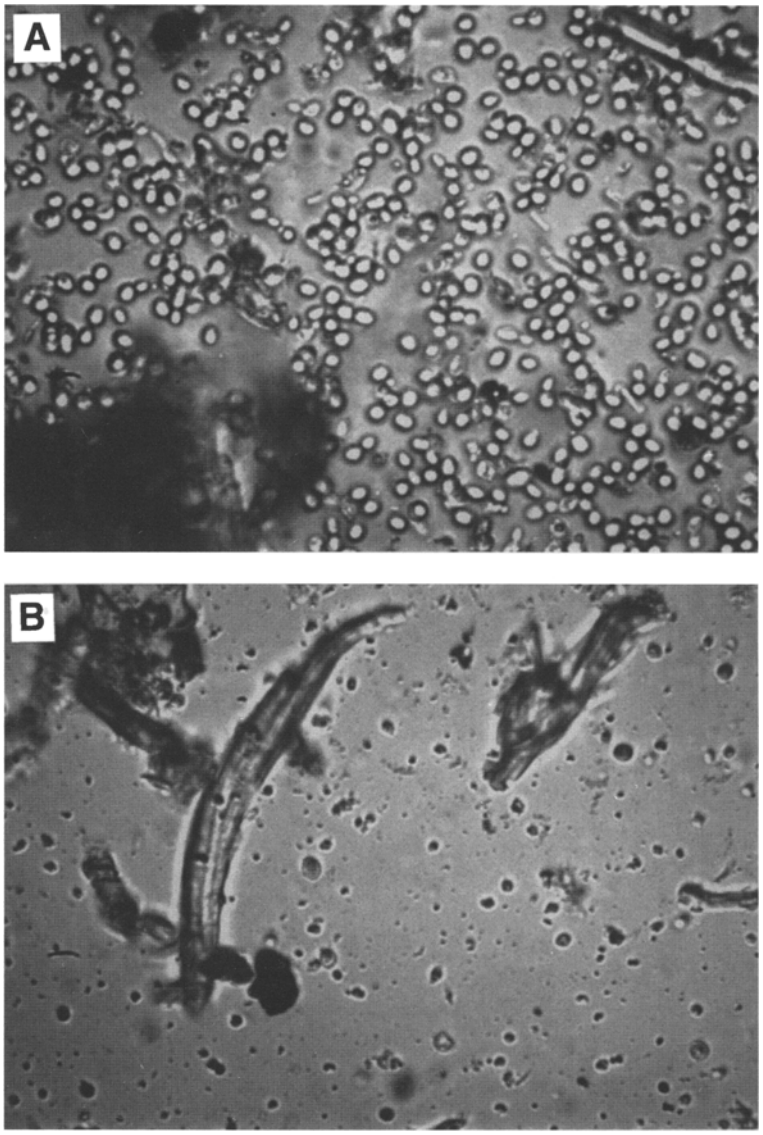

Fig. 4. Photomicrograph (viewed at 400X) of yeast cells (0.3 wt%) with acid-pretreated switchgrass (3 wt%); **(A)** prior to treatment with lysing enzyme; **(B)** following treatment with enzyme.

plus cobs) substrates is summarized in Table 1. The cellulose content of each SSF residue was measured with and without inclusion of the enzymatic lysing step for removal of yeast glucans. The actual amount of cellulose in the 7 d SFF residues was 773 mg (29% of original), 389 mg (14% of original), and 361 mg (12% of original) for poplar, switchgrass, and cornstover, respectively. Failure to remove yeast-associated glucans from the SSF residues

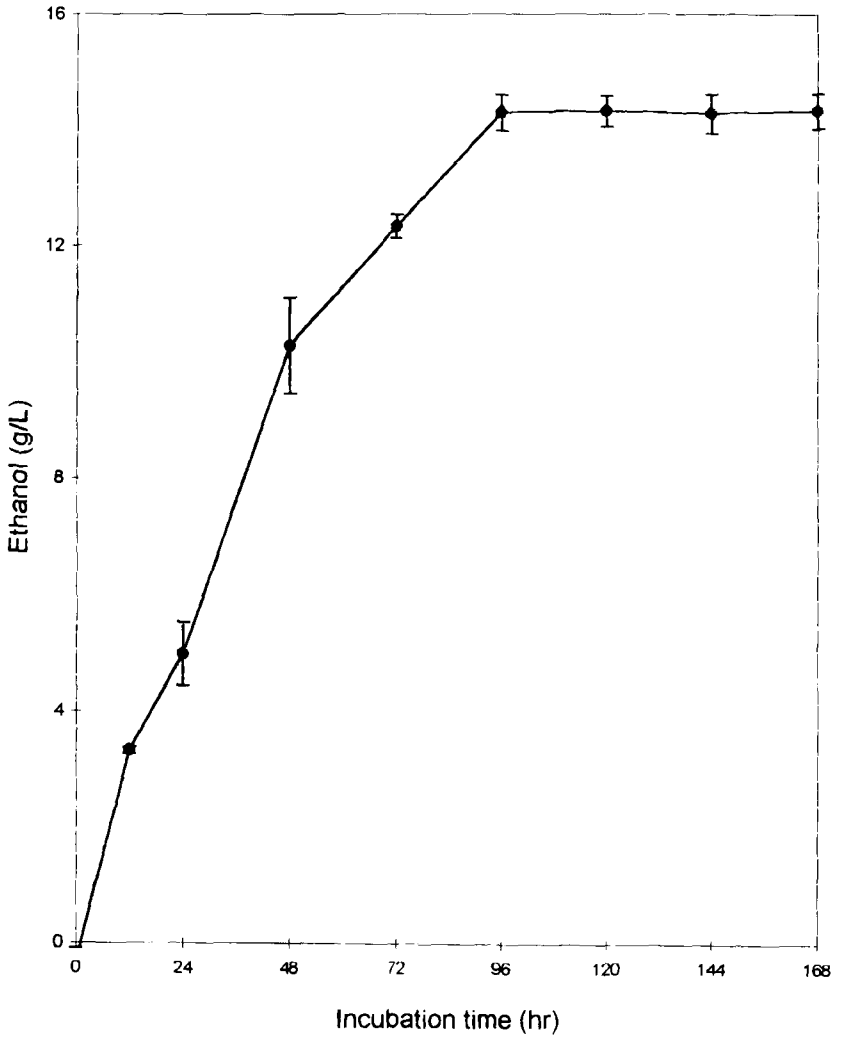

Fig. 5. Time-course for ethanol production in simultaneous saccharification and fermentation experiments with pretreated switchgrass. Reaction/fermentation mixtures contained approx 6 wt% pretreated switchgrass (equivalent to approx 3.0 wt% cellulose). Data are presented as the mean and standard deviation (error bars) of two assays.

prior to cellulose analysis resulted in a relative error of 4.1, 2.8, and 7.2% for the poplar, switchgrass, and cornstover substrates, respectively. The error is always associated with an overestimation of the amount of cellulose remaining in the residue. The magnitude of the error will increase as the number of yeast cells increases and the amount of residual cellulose decreases, as may be expected toward the end of an SSF experiment. The magnitude of the error is likely to increase in proportion to the effectiveness of the pretreatment that the feedstock undergoes prior to the SSF experiment, because the severity of the pretreatment is known to directly affect both the rate

and extent of cellulose saccharification *(13,14)*. The difference in the two measured cellulose values for a given substrate (measured with and without the use of lysing enzyme) is equivalent to the amount of yeast-derived glucan in that SSF residue. The values generated in this study may be used by others to gage the potential relevance of yeast glucans under their particular experimental conditions. The absolute amount of yeast-derived glucose equivalents associated with the poplar, switchgrass, and cornstover residues following 7 d SSF were 32, 11, and 26 mg, respectively.

The concept and application of using lysing enzyme to more accurately determine the cellulose content of SSF residues has been the primary focus of this article. However, this same approach is valuable for other aspects of SSF-residue analysis. For example, it is often of interest to follow changes in the physical properties of particulate substrates as SSF experiments progress. A technique that can be used to gather information on particle size and shape makes use of optical microscopy-image analysis systems. In using these systems, it is sometimes necessary to remove yeast cells from the SSF residue so that the microscopic image to be analyzed is limited to the particulate substrate and not a mixture of substrate particles and yeast cells. Similarly, the accuracy of some of the techniques used for the measurement of residue surface area are dependent on the removal of yeast solids.

In conclusion, the commercially available lysing-enzyme preparation from *Cytophaga* was found to be appropriate for use in removing yeast-cell glucans from SSF residues, thus allowing for more accurate determination of the cellulose content of these residues. Lysing-enzyme activity was shown to be affected by the presence of biomass substrates and, hence, an experimental protocol was suggested for the determination of the minimum incubation time necessary to insure the complete removal of yeast glucans from a given type of SSF residue. Yeast cells in the latter phases of SSF experiments were shown to be resistant to lysing enzyme. However, this resistance could be overcome by freeze-drying the SSF residue prior to enzyme treatment.

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