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OPTIMUM β-D-GLUCOSIDASE SUPPLEMENTATION OF CELLULASE FOR EFFICIENT CONVERSION OF CELLULOSE TO GLUCOSE

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SUMMARY

To assess optimal saccharification performance, α-cellulose and dilute acid pretreated aspen (DAA) wood meal were subjected to various loadings of commercial cellulase and β-D-glucosidase preparations. Fifteen international filter paper units (IFPU)/g cellulose content and 30 IFPU/g cellulose content were required to digest 95% of the available cellulose in α-cellulose and pretreated aspen, respectively. The optimal supplementation ratios, based on Genencor GC 123 cellulase and β-D-glucosidase from Novo SP188 for the α-cellulose and DAA digestions range from 0.25 to 0.5 and 0.12 to 0.25, respectively.

INTRODUCTION

Cellulase enzymes act on cellulose to produce glucose by cleaving the β-1,4 glucosidic linkages in the polymer chain. With fungal cellulases, this saccharification is enhanced by supplementation with β-D-glucosidase (EC 3.2.1.21), which hydrolyzes cellobiose selectively to glucose (King and Vessal, 1976; Wood and McCrae, 1981). Cellobiose accumulation is known to inhibit the enzymatic activity of both the cellobiohydrolase and endoglucanase components of the fungal cellulase complex (Gritzali and Brown, 1979; Ryu and Mandels, 1980).

The cost of cellulase enzymes and the efficiency of the saccharification process is of great interest not only for separate hydrolysis and fermentation (SHF) applications (Wright et al., 1986), but also for simultaneous saccharification and fermentation (SSF) processes (Wilke et al., 1976; Wright et al., 1988). In SSFs, ethanol is produced by concurrent saccharification and yeast fermentation, utilizing the glucose produced from the hydrolysis of the cellulosic content in biomass. Ethanol must be produced at a competitive price to be useful as a renewable liquid fuel.

This study focuses on the determination of the optimum ratio of cellulase enzymes and β-D-glucosidase for the production of glucose from dilute acid pretreated aspen meal. This substrate represents a "real" lignocellulosic substrate from the large-scale process perspective and as such was considered of more interest than typical cellulose model

substrates like Avicel and Solka-floc. Each cellulose digestion was augmented with 4 different concentrations of a commercial β-D-glucosidase preparation to further investigate the optimum β-D-glucosidase/cellulase ratio (supplementation ratio) for these substrates. Conditions delineating the highest yield of glucose with the lowest usage of enzymes were targeted in this study.

MATERIALS AND METHODS

Substrates, enzymes, and reagents. The α-cellulose and the p-nitrophenyl-β-D-glucoside (pNPG) used in this study were obtained from Sigma Chemical Company. The pretreated aspen meal was prepared from aspen wood that had been knife-milled to 60 mesh and subjected to dilute acid hydrolysis following the method of Grohmann et al. (1985). The cellulase enzyme preparation used was the GC 123 Cellulase from Genencor, Inc., South San Francisco, Calif. and the β-D-glucosidase preparation was the Novozym SP188 (lot 1005) from Novo Labs, Danvers, Ohio. All buffer components and salts used were reagent grade and obtained from either Sigma Chemical Company or Fisher Scientific.

Enzyme assays. Assays for saccharifying cellulase (i.e., international filter paper units, IFPU) activities followed the methods recommended in the 1987 IUPAC report (Ghose, 1987). β-D-Glucosidase was determined according to the method of Wood (1981) as aryl-β-glucosidase activity by the hydrolysis of p-nitrophenyl-β-D-glucopyranoside. The concentration of p-nitrophenol (pNPOH) was determined from the extinction at A_{410} under alkaline conditions induced by the addition of 2 M Na_2CO_3. One unit of activity was defined as that amount of enzyme that catalyzes the cleavage of 1.0 μmol substrate per minute at 45°C.

Cellulose digestion protocols. Substrate slurries were prepared to a concentration of 50 g/L in 20 mM acetate buffer, pH 4.8 and stabilized with tetracycline and cycloheximide (40 μg/mL and 30 μg/mL, respectively). These slurries were placed in polyethylene screw-capped centrifuge tubes and incubated in the presence of various enzyme loadings at a controlled temperature of 45°C. The samples were rotated at approximately 2 rpm during the incubation period. Samples were removed periodically from each tube for analysis and care was taken to remove representative aliquots (liquid and solids) for each 24-h time point. Glucose was determined using a YSI model 27 glucose analyzer (Yellow Springs Instruments, Yellow Springs, Ohio). Before glucose analysis, the aliquots were diluted with distilled water and boiled for 5 min to inactivate enzyme activity.

α-Cellulose digestion. α-Cellulose (2.5 g) was added to 50 mL of buffer for an effective cellulose loading of 2.37 g (i.e., this substrate was 95% w/w cellulose), or 4.8% cellulose solids. Enzyme loading to the tubes were designed so that the GC 123 cellulase to SP188 β-D-glucosidase ratios, units pNPG hydrolyzing activity/IFPU, were 0, 0.25, 0.50, 1.0, and 2.0. Cellulase concentrations used were 5, 10, 15, 20, 25, and 40 IFPU/g cellulose content.

Pretreated aspen meal. The dilute acid treated aspen sample was found to be approximately 63% w/w cellulose in previous studies (Grohmann et al., 1986); therefore, 3.8 g of the substrate was added to 50 mL of buffer in each tube to produce a 4.8% cellulose solids slurry. In addition to the six cellulase loadings used in the α-cellulose control described above, the aspen substrate digestion included tubes loaded with 30, 60, and 80 IFPU/g cellulose content. These extra loadings provided increased data density at the apparent enzyme saturation levels.

RESULTS AND DISCUSSION

This study examined the performance of GC 123 Cellulase, a commercial cellulase preparation from Genencor, Inc., on the digestibility of both α-cellulose and pretreated

aspen wood meal. These studies were performed in the presence of various concentrations of β-D-glucosidase obtained by the introduction of aliquots of Novo SP188, also a commercial preparation. The Genencor GC 123 and Novo SP188 preparations were found, by the assays given above, to contain 90 IFPU/mL and 250 pNPGU/mL, respectively. Following conventional Lowry protein assays, the preceding values can be converted to 980 IFPU/g Lowry protein and 1800 pNPGU/g Lowry protein, respectively. Genencor GC 123 was also found to have 45 pNPGU/mL β-D-glucosidase activity.

Although GC 123 is the most recent cellulase preparation from Genencor, it was found to contain lower cellulase activity than the previous preparation, Genencor 150L (i.e., 90 IFPU/mL for GC 123 compared to 106 IFPU/mL for 150L), and a lower pNPGU/IFPU ratio than Genencor 150L (i.e., 0.50 for GC 123 compared to 1.51 for 150L)(Spindler et al., 1989). This decrease in filter paper activity is most commonly attributed to this dramatically lower β-D-glucosidase/cellulase ratio.

The primary data for these studies were used to construct plots of the percentage of digestibility versus digestion time. These plots (not shown) were constructed for each cellulase/β-D-glucosidase combination for both substrates, representing 70 separate digestions, most of which were performed in duplicate. The maximal percentage digestibility value, taken as that reached after 7 days of incubation, was obtained from smoothed versions of these digestion time-course plots. In most cases, the production of glucose was complete by 5 days of incubation.

Figures 1 and 2 show not only the effects of cellulase loadings on the digestibility of α-cellulose and pretreated aspen wood, respectively, but also the synergistic effects of β-D-glucosidase supplementation. Although the diminishing effect of cellulase loading is apparent from the relative features of the curves shown in Figures 1 and 2, Figure 3 shows the digestibility breakpoints more graphically. As shown in Figure 3, the pretreated aspen wood substrate demonstrates a less abrupt transition from low levels of digestibility to complete (or near complete) saccharification than does the less compositionally complex α-cellulose. This observation may be due to the well-documented loss of cellulase enzymes (especially β-D-glucosidase) through adsorption to lignin (Sutcliffe and Saddler, 1986; Tatsumoto et al., 1988). Further examination of Figure 3 reveals that 15 IFPU/g cellulose for α-cellulose and 30 IFPU/g cellulose for pretreated aspen wood loadings produce 95% of the maximal saccharification found for each substrate under these experimental conditions, i.e., 45°C, 4.8% cellulose solids, and gentle mixing. The maximal saccharification found for α-cellulose and pretreated aspen wood was 91% and 88%, respectively.

The effect of β-D-glucosidase supplementation is also shown in Figures 1 and 2. The optimal supplementation ratio for the α-cellulose and pretreated aspen wood digestion ranges from 0.25 to 0.5 and 0.12 to 0.25, respectively. Rather than reporting the effects of mixing pure enzyme mixtures, these results show more directly the effects of blending

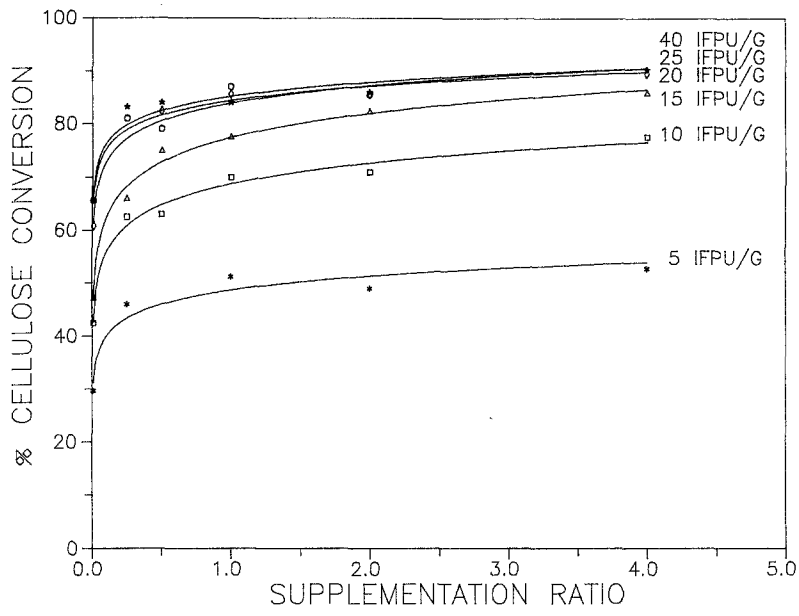

Figure 1. Effects of GC 123 cellulase loading and SP188-derived β-D-glucosidase/cellulase supplementation ratio of the saccharification of α-cellulose at 45°C, pH 4.8, and 4.8% cellulose solids. Supplementation ratios given do not reflect the β-D-glucosidase contributed by GC 123 (add 0.5 to each value for this correction).

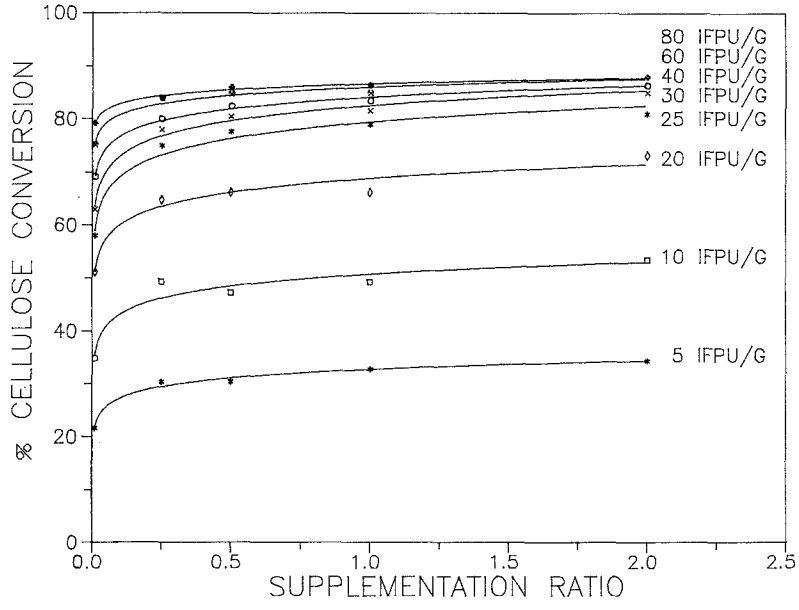

Figure 2. Effects of cellulase loading and β-D-glucosidase/cellulase supplementation ratio of the saccharification of dilute acid pretreated aspen wood meal at 45°C. pH 4.8, and 4.8% cellulose solids.

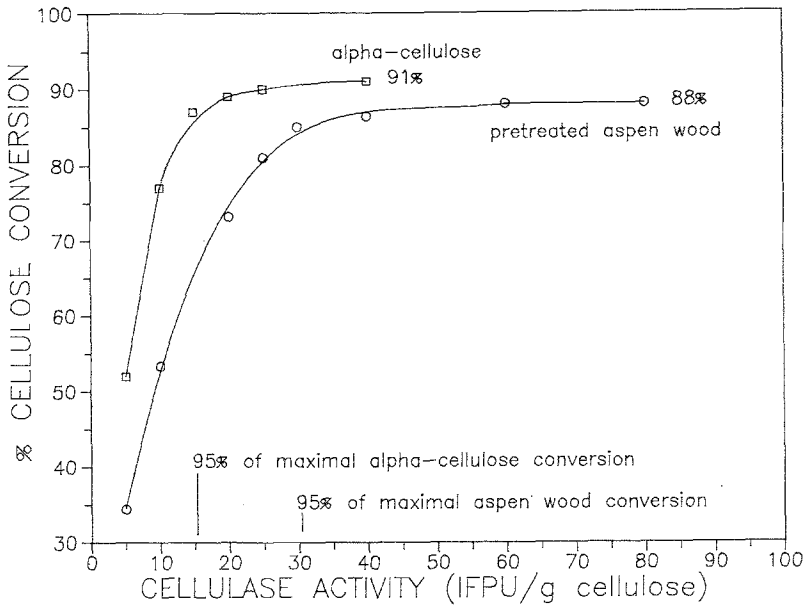

Figure 3. Relationship between the greatest obtainable saccharification of the cellulose content of each substrate and the corresponding cellulase enzyme loading (taken at a supplementation ratio of 4).

the Genencor and Novo industrial preparations. In fact, the results presented in Figures 1 and 2 present the case of "0" supplementation as the saccharification system with no added β-D-glucosidase; however, the level of pNPG hydrolyzing activity found in Genencor GC 123 was found to be approximately one-half the concentration of the filter paper degrading activity. Therefore, the supplementation ratios shown in Figures 1 and 2 will be increased by 0.5 when total β-D-glucosidase loading is considered.

These values describe qualitatively the synergistic effect of β-D-glucosidase supplementation under the experimental conditions tested. It is likely that changes in temperature, substrate, enzyme source, and solids concentration will affect the optimum supplementation requirements. However, the eventual application of these enzyme preparations to SSF, where optimum performance is measured by ethanol production, should generally reflect required enzyme concentrations that are less than those found optimal from cellulose saccharification studies, because of relief of the well-documented substrate inhibition effects in SSF. This assumption was supported preliminarily by the determination from Spindler et al. (1988), using the obsolete Genencor 150L, that 13 IFPU/g cellulose was optimal for the production of ethanol from the SSF of α-cellulose. These results agree well with those from the present study where 15 IFPU/g cellulose was considered optimal for the saccharification of α-cellulose using the current preparation of Genencor cellulase, GC 123.

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