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Cellulase Production on Bagasse Pretreated with Hot Water

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Abstract

Because pretreatment of biomass with hot water only in differential flow systems offers very digestible cellulose and potentially less inhibition by liquid hydrolysate, solids and liquid hydrolysate from bagasse pretreated with hot water were fed to a batch cellulase production system using the Rut C30 strain of *Trichoderma reesei* to determine the suitability of these substrates for cellulase production. The organism was found to be sensitive to inhibitors in the liquid hydrolysate but could be adapted to improve its tolerance. In addition, filtering of the material reduced inhibitory effects. The organism was also sensitive to some component in the solids, and they had to be washed heavily to achieve good growth and cellulase production rates. Even then, a lag was found before enzyme production would commence on pretreated solids whereas no such lag was experienced with Solka Floc. However, once enzyme production began, as high and even somewhat greater cellulase productivities were realized with washed pretreated solids. Adding lignin to Solka Floc delayed enzyme production, suggesting that lignin or other materials in the lignin solids could cause the lag observed for pretreated bagasse, but more studies are needed to resolve the actual reason for this delay.

Index Entries: cellulase production; hot water pretreatment; Rut C30; *Trichoderma reesei*.

Introduction

Cellulosic biomass such as agricultural (e.g., corn stover, sugarcane bagasse) and forestry (e.g., pulp mill sludge) residues, municipal solid waste (particularly waste paper), and dedicated crops (e.g., switchgrass and hybrid poplar) provides a low-cost feedstock for biologic production of a wide range of fuels and chemicals that offer substantial economic,

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environmental, and strategic advantages *(1)*. Cellulose and hemicellulose comprise about two thirds to three quarters of these materials, and through the application of modern genetics and other tools from the rapidly advancing field of biotechnology, the cost of producing sugars from these recalcitrant fractions and converting them into products such as ethanol has been significantly reduced. However, additional cost reductions are needed to achieve competitiveness with conventional fuels.

The use of cellulase enzymes is the most promising approach to realizing the high product yields vital to economic success *(2,3)*, but cellulase production and application are currently among the more costly processing steps for biologically based routes to bioethanol production *(3–5)*. Fortunately, these steps also offer the greatest potential for cost reduction through application of rapidly emerging advances in the fields of modern biotechnology and bioprocessing *(2)*. The use of waste and other low-cost sources of cellulosic biomass, instead of lactose or other more expensive inducers/substrates currently employed commercially for limited high-value specialty markets, would significantly reduce substrate costs for cellulase production. However, enzyme productivities are significantly lower on cellulose than for lactose in solution *(6,7)*, and larger vessels are needed when cellulose is the carbon source. In addition, power costs for mixing and aeration increase significantly owing to the greater volumes and higher viscosities that result with enzyme production on cellulose. Thus, costs could be reduced by increasing cellulase productivity on pretreated biomass. Increasing cellulase yields and specific activity would also lower feedstock costs, and increasing enzyme titer would reduce the amount of water needed to process with the enzyme *(4)*.

Cellulosic biomass must be pretreated to achieve high glucose yields *(8)*, and various studies have shown that pretreatment with water passing through solids at high temperatures in some type of differential flow system removes hemicellulose sugars as oligomers with high yields *(9,10)*. Furthermore, the cellulose residue is more digestible by enzymes than cellulose from conventional dilute-acid technologies, possibly owing to greater lignin removal, and the liquid hydrolysate is also less toxic to the growth of organisms *(11)*. Thus, biomass pretreated by hot water flow systems could accelerate the rate of organismic growth and enzyme production compared to other sources of pretreated cellulose studied in the past and reduce the enzyme loading required to achieve a given cellulose hydrolysis yield. Based on this reasoning, the present study focused on a preliminary evaluation of cellulase enzyme production using biomass solids after pretreatment with hot water in a differential residence time system to assess whether this route could enhance performance on inexpensive biomass and reduce enzyme production costs. Additional goals were to evaluate whether the hemicellulose oligomers in the hydrolysate from hot water pretreatment would promote production of an enzyme cocktail that hydrolyzes hemicellulose oligomers and cellulose and to determine

whether the toxicity was low enough to minimize, if not eliminate, the need to detoxify the substrate for cellulase production.

Materials and Methods

Feedstocks

The carbon sources used to support fungal growth and cellulase production were filter-sterilized sorbitol, Solka Floc 40 from Fiber Sales and Development Corporation (Eastech Chemical, Inc, Philadelphia, PA), the liquid hydrolyzate from pretreatment of Hawaiian sugarcane bagasse, and the solid residue from the same pretreatment operation. The University of Hawaii produced the latter two fractions in their liquid hot water pretreatment system for a 2 min processing time at 220 to 230°C, as described by Allen et al. *(12)*. The conditions were selected based on performance measured for similar systems *(12,13)*. The pretreated damp solids were subjected to three primary washes with tap water and three secondary washes with distilled water. In each wash, the material was mixed with 10 times its weight of water for 30 min using a magnetic stirrer. The washed material was centrifuged for 20 min, and the liquid decanted.

Cellulase Producing Organism

The Rut C-30 strain of *T. reesei* was obtained from the National Renewable Energy Laboratory (NREL) for these experiments. The organism was stored as spores that were formed by growing *T. reesei* to maturity on potato dextrose plates. The resulting spores were harvested by swirling in a small amount of sterile distilled water on the plates and combining 0.8-mL aliquots with 0.2 mL of glycerol in freeze vials to be frozen at –80°C. A seed train protocol was used to ensure that growth conditions were the same for all experiments. First, frozen spores were grown on 20 g/L of glucose for 48 h in shake flasks, and then the organism from this step was transferred as a 5% (v/v) inoculant into a seed flask containing standard shake-flask medium with either 10 g/L of Solka Floc or 10 g/L of sorbitol depending on the substrate used in the experiment. The seed flask was grown for 48 h and used as the inoculum for our cellulase production process.

Culture Methods

The methods employed were based on standard procedures to the maximum extent possible. We particularly relied on protocols developed by NREL *(14)* and advice by NREL on the application of these procedures. Shake-flask experiments were conducted in 500-mL baffled shake flasks with a 100-mL culture volume. Each flask contained the following components: 50 mL of Mandel's medium, 1 mL of trace minerals, 10 mL of phthalate buffer, 0.02 mL of Tween-80 carbon source, 5% (v/v) seed culture, and enough distilled water to realize a final total volume of 100 mL. The Mandel's medium contained 0.4 g/L of $CaCl_2 \cdot 2H_2O$, 0.3 g/L of $MgSO_4 \cdot 7H_2O$,

2.0 g/L of KH_2PO_4, 1.4 g/L of $(NH_4)_2SO_4$, 5.0 g/L of peptone, and 5.0 g/L of yeast extract. Trace mineral stock (100X) was made up of 0.5 g/L of $FeSO_4 \cdot 7H_2O$, 0.016 g/L of $MnSO_4 \cdot H_2O$, 0.014 g/L of $ZnSO_4 \cdot 7H_2O$, and 0.37 g/L of $COCl_2 \cdot 6H_2O$, and the phthalate buffer was a 0.5 *M* potassium hydrogen phthalate solution. The pH was adjusted to 4.8 and the material filter sterilized. Silicone sponge closures (part 2004-00005; Bellco Glass, Vineland, NJ) were used to cap the flasks.

Analyses

Samples were drawn from flasks regularly using wide-bore pipetes. The samples from cultures containing solid substrate were immediately spun down, and the solids and supernatant stored separately at 5°C. The supernatant was later analyzed for protein and/or cellulase activity.

Optical density (OD) was used to estimate cell growth on soluble substrates by measuring absorbance at 660 nm on a Milton Roy Spectronic 21D spectrophotometer, and protein was determined by Bio-Rad Protein Assay Kit II, which contains lyophilized bovine serum albumin (BSA) standard (part no. 500-0002). Cellulase activity was determined by the standard filter paper assay described in NREL LAP-006 "Measurement of Cellulase Activities" *(14)*. Quantitative saccharification following the LAP-002 protocol was employed to measure the carbohydrates in the solids *(14)*. The digestibility of the pretreated solids was determined from the yield of ethanol in standard simultaneous saccharification and fermentation experiments as described in LAP-008 *(14)*.

Results

Bagasse feed for cellulase production was pretreated at conditions that gave high yields of hemicellulose sugars, as determined in previous work *(12,13)*; the total concentration of sugar monomers and oligomers in the liquid hydrolysate are reported in Table 1. The liquid and solid fractions were then evaluated for cellulase production.

Cellulase Production on Liquid Hydrolysate

The first experiments focused on the liquid hydrolysate from pretreated bagasse. A series of cultures were grown on combinations of hydrolysate and 10 g/L of sorbitol as the carbon source, varying from 0 to 80% hydrolysate. In this context, a medium described as 50% hydrolysate designates a mixture for which 50% of the distilled water component of the medium was replaced with hydrolysate. Using pure hydrolysate as a benchmark, we normalized sugars to 1.4 g/L of xylose, 0.2 g/L of arabinose, and 0.1 g/L of glucose. No significant inhibition was observed in mixtures ranging from 0 to 15% hydrolysate, but at the next level, 40% hydrolysate, initial growth was delayed by approx 22 h. Interestingly, despite the initial lag, growth caught up with the 15% culture at about 40 h, but cultures with 50% hydrolysates (not shown) did not grow at all. Figure 1 summarizes these results.

Table 1
Characterization of Liquid Hydrolysate and Solid Residue from Pretreatment at 220 to 230°C for 2 min

Solids analysis	
Cellulose (%), dry wt basis	68.6
Liquid analysis	
Sucrose	ND[a]
Arabinose (g/L)	0.154
Galactose (g/L)	0.055
Glucose (g/L)	0.057
Xylose (g/L)	1.382
Mannose (g/L)	0.002
Fructose (g/L)	0.014
Ribose (g/L)	0.005
Total soluble sugars (g/L)	1.669

[a] ND, not determined.

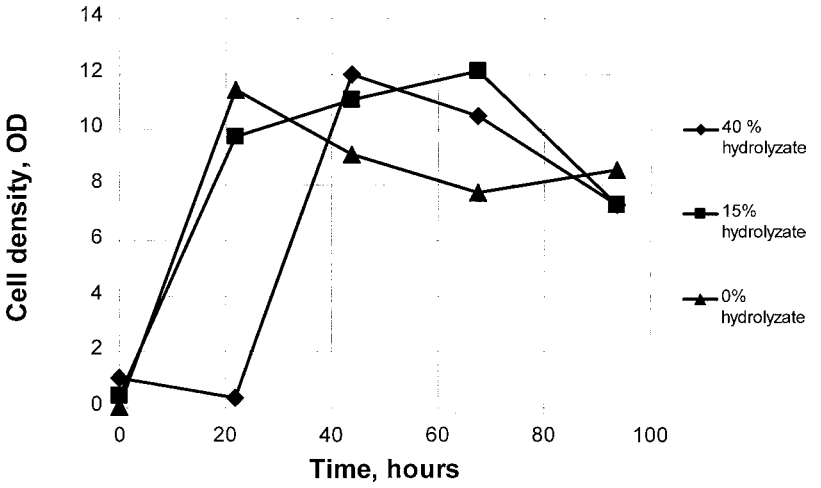

Fig. 1. The effect of hydrolyzate concentration on cell density.

Because the hydrolysate used for all cultures was autoclaved with the base medium, experiments were conducted to examine whether autoclaving was increasing the toxicity of the hydrolysate. Using 50% hydrolysate as the test level, we prepared flasks containing hydrolysate that was autoclaved in the flask with base medium (the control), filter sterilized only and added to a flask containing sterile base medium, and first filter sterilized and then autoclaved in the flask with the base medium. The filter-sterilizing protocol consisted of three filtering steps: filtering the original material through Whatman #1 filter paper, filtering the product from step 1 through 0.4-μ filters, and passing the resulting material through 0.2-μ

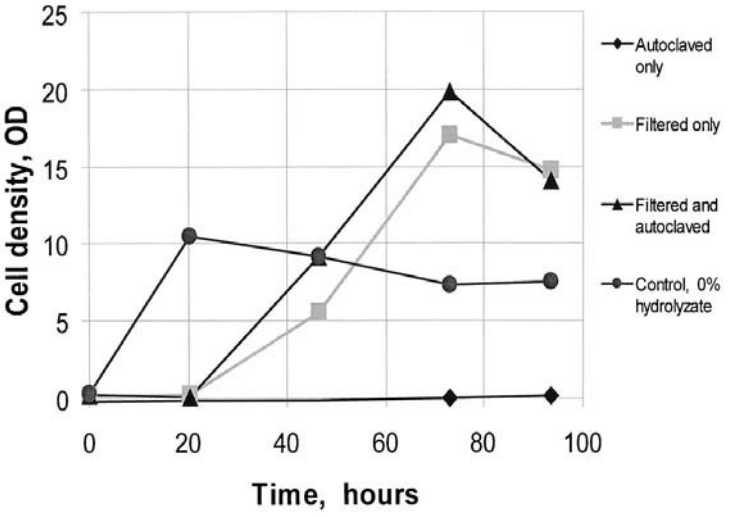

Fig. 2. The impact of filtration and autoclaving on growth of Rut C30 cells over time.

filters. Figure 2 summarizes the results for the filtration studies. No growth was observed for the autoclaved hydrolyzate, just as before. However, inhibition was much less for cultures grown on filter-sterilized hydrolysate. In fact, these cultures grew vigorously following an initial lag, and filtration greatly reduced the inhibition by autoclaved hydrolysate even though filtration preceded autoclaving. The filtering process apparently removes toxins or those constituents that become toxic in the autoclave.

Next, we evaluated whether Rut C30 could be conditioned to grow on higher proportions of hydrolysate by growing the inoculant on medium containing 10 g/L of sorbitol plus 30% hydrolysate for 12 d. When this adapted organism was transferred to the primary enzyme production shake flasks, the growth rate with 40, 50, and 60% hydrolysate was better than for the control without hydrolysate conditioning while no growth was observed for the 80% hydrolysate (Fig. 3). However, the rate of growth decreased as the amount of hydrolysate was increased beyond 40% hydrolysate. Although the results from conditioning were encouraging, tolerance to hydrolysate was lost in spores, and it appeared to be necessary to maintain a conditioned culture by serial transfer, with one culture inoculating the next. This approach is difficult to maintain, may be vulnerable to inadvertent selection for irrelevant and even detrimental traits, and could prove susceptible to contamination. In addition, the conditioned strains were not found to be good cellulase producers by NREL researchers, who provided us with helpful advice on this project. Based on these considerations and time limitations, we decided not to pursue conditioning of *T. reesei* further.

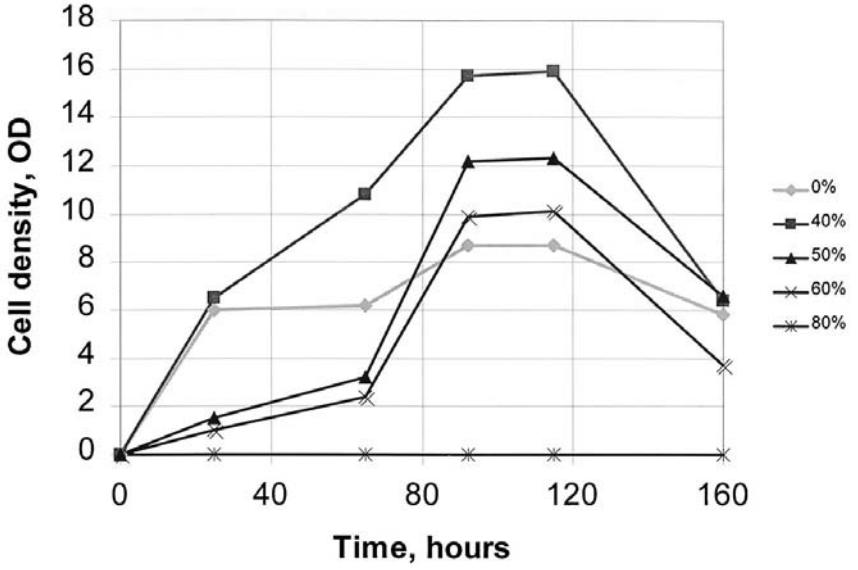

Fig. 3. The effect of hydrolyzate strength on growth of conditioned Rut C30.

Enzyme Production on Pretreated Solids

Enzyme production experiments were run over a range of cellulose concentrations from 5 to 25 g/L and compared to control experiments with Solka Floc at a cellulose concentration of 10 g/L. The FPA requires a relatively large (5- to 6-mL) sample, and taking daily FPA samples would seriously reduce the reaction volume. Therefore, we supplemented the FPA data with daily BSA protein assays that require much smaller samples to provide a more comprehensive picture of the protein production profile.

In initial experiments, solids were washed three times with distilled water, as described previously before addition of base medium and autoclaving. However, varying degrees of lag in initial growth followed by relatively weak growth were observed. As shown in Fig. 4, more extensive washing of the pretreated solids resulted in vigorous growth, although an initial lag could not be eliminated. The lag could be owing to initial adsorption of cellulase on lignin and cellulose. In addition, as noted earlier, the liquid hydrolysate appeared to contain compounds that reduced the growth rate after the lag phase, and the higher growth rate with heavily washed solids could be owing to removal of these components.

Figure 5 presents the protein production profile for a typical experiment consisting of three replicates with washed pretreated bagasse and two replicates for growth on Solka Floc. All are for a concentration of 10 g/L of cellulose, and the variation in results between replicates is small. By compiling data from four different shake-flask experiments, it appeared that the protein productivity was almost identical for pretreated solids and Solka Floc after about 120 h (Fig. 6).

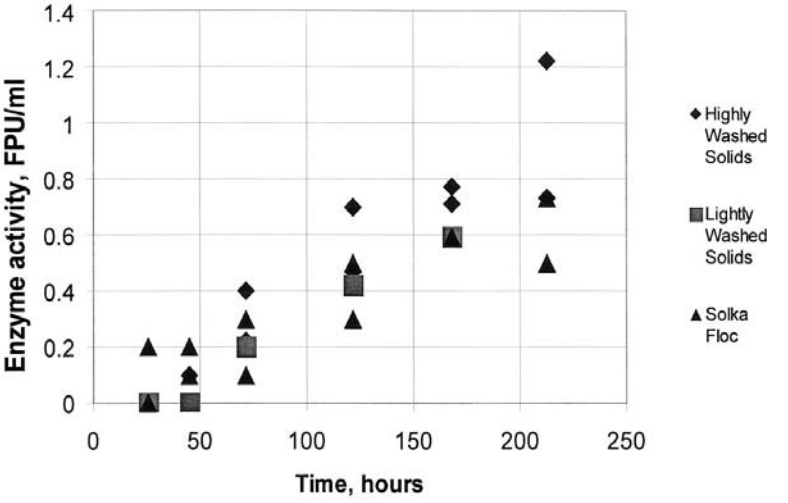

Fig. 4. Enzyme production profiles with varying solids washing.

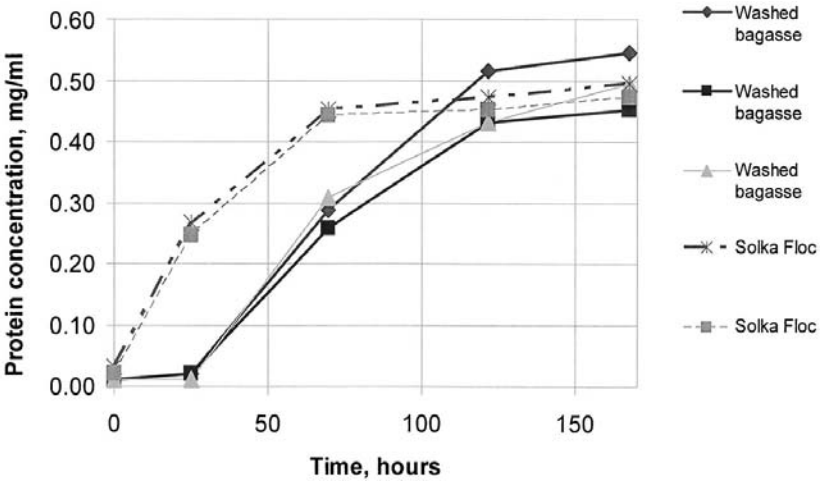

Fig. 5. Protein production profiles for repeated runs with washed pretreated solids and Solka Floc.

Rut C30 generally produced between about 0.40 and 0.75 filter paper units (FPU)/mL over approx 168 h when grown on 10 g/L pretreated bagasse and between about 0.5 and 0.7 FPU/mL when grown on Solka Floc at the same conditions (Figs. 7 and 8). Based on limited FPA measurements collected over three experiments for pretreated solids, cellulase productivity averaged 3.1 FPU/(L·h) after approx 168 hours and 2.9 FPU/(L·h) for Solka Floc. Thus, although the lag for pretreated bagasse reduced productivities at shorter times, the productivity was at least as great for pretreated solids once enzyme production commenced. Analysis of the solid residue from shake-flask runs by the quantitative saccharification

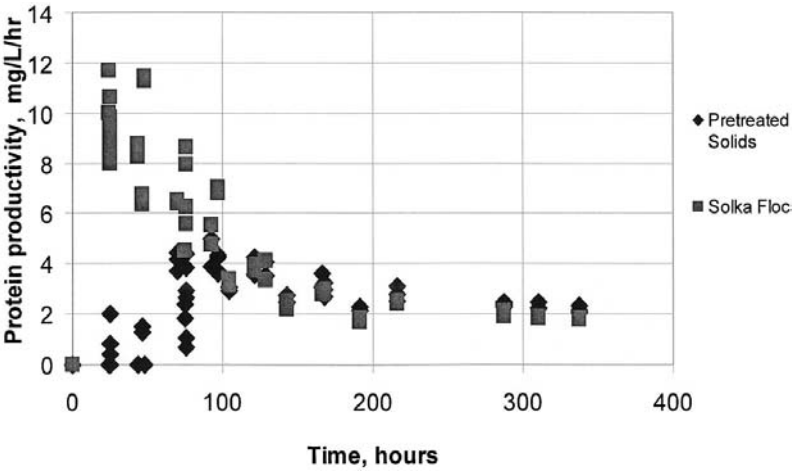

Fig. 6. Comparison of protein productivity for pretreated bagasse solids and Solka Floc.

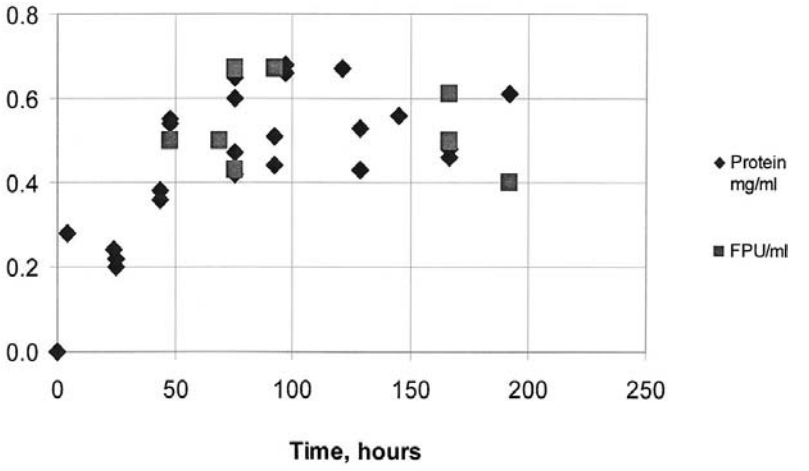

Fig. 7. Cellulase activity and protein concentration profiles for Solka Floc control at 10 g/L cellulose.

protocol revealed that 98% of pretreated solids and 99% of Solka Floc controls were converted at 168 h.

A few experiments were run to estimate the effect of cellulose concentration on enzyme production, as summarized in Table 2, and the organism appeared to perform somewhat better with increasing concentrations of Solka Floc than for higher levels of pretreated solids. This effect could be owing to the inhibitory effects of the pretreated solids observed earlier, but further studies are needed to verify this relationship.

Both the Solka Floc controls and pretreated bagasse solids described in the previous experiments were run at the same cellulose concentrations.

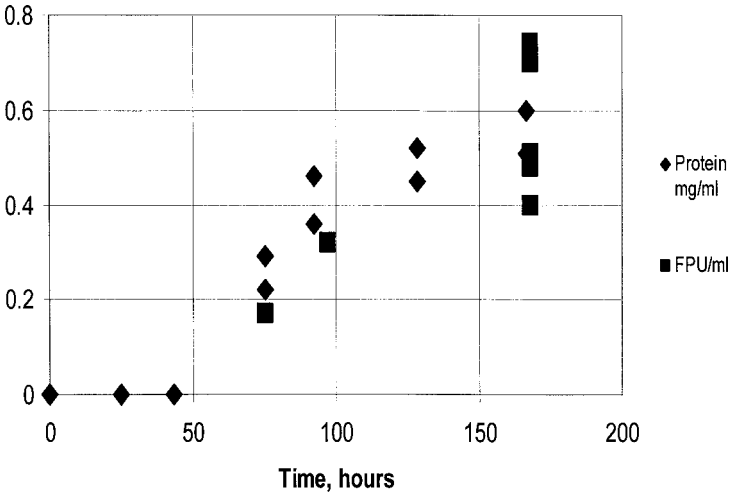

Fig. 8. Cellulase activity and protein concentration profiles for pretreated bagasse solids at 10 g/L cellulose.

Table 2
Effect of Cellulose Concentration on Cellulase Production for Pretreated Bagasse Solids and Solka Floc

	Protein (mg /mL)		FPU/mL	
Cellulose (g/L)	Pretreated bagasse solids	Solka Floc	Pretreated bagasse solids	Solka Floc
5	0.50	0.29	0.3	0.3
10	0.55	0.48	0.6	0.6
15	0.55	0.90	—	—
25	—	—	0.6	0.8

However, whereas Solka Floc is nearly 100% cellulose on a dry wt basis, the pretreated bagasse contained about 69% cellulose with the remainder being lignin and low levels of other solids such as ash. As a result, the overall concentration of solids for pretreated bagasse is about 45% higher than for the Solka Floc controls in the cellulase production experiments. To determine whether the higher-fraction solids could reduce mixing and impede oxygen transfer for the aerobic growth of *T. reesei*, lignin-rich material was added to Solka Floc to increase the solids concentration beyond that for pretreated bagasse. Raw, chopped, dried bagasse was treated with 72% (w/w) H_2SO_4 for two hours at 30°C, as in the quantitative saccharification procedure. The solid residue was washed very thoroughly until a neutral pH was achieved, and then it was dried and combined with Solka Floc to produce a solid substrate with a cellulose concentration equal to that of the control. However, the overall solids

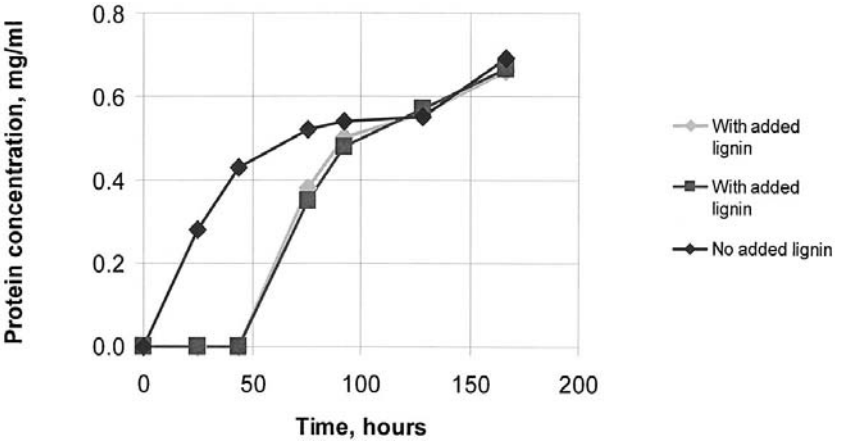

Fig. 9. Protein production profiles for Rut C30 grown on Solka Floc only and for two repeated runs with lignin addition.

concentration was now 150 g/L compared with only about 14.5 g/L for pretreated bagasse solids. As shown in Fig. 9, protein accumulation with duplicate mixtures of Solka Floc and lignin was delayed for 2 d, but then produced enzyme at a similar rate to using Solka Floc alone. This pattern of protein production was similar to that observed with pretreated bagasse solids.

The substantial lag and its enhancement with increasing lignin levels strongly suggest that lignin delays enzyme production and could account for the lag observed with the pretreated solids. To shed light on this hypothesis, a new culture was grown in a flask containing 20 g of glucose, 2.5 g of lignin, and 5 mL of phthalate buffer in a volume of 50 mL, for an overall solids concentration of approx 70 g/L. After 48 h, growth on this mixture was very poor whereas the culture in a control flask grew normally and the pH stayed well within the normal working range. This is a further indication that lignin delays organismic growth, perhaps due to inhibitory effects that exceed any effects caused by increased solids concentration or by greater adsorption of enzyme.

To ensure that oxygen transfer was not limiting cellulase production rates, both Solka Floc and pretreated bagasse were used for enzyme production at concentrations of 10 g/L of cellulose with and without the addition of oxygen. In these screening experiments, oxygen was bubbled through distilled water and delivered through diffusers resting on the bottom of the shake flasks with the shaker table speed set at 200 rpm. Despite bubbling gas through water, evaporation was significant for the oxygenated flasks (as high as 45% over the course of these experiments); therefore, the protein production results reported in Fig. 10 were corrected for the loss of volume resulting from evaporation. Although the initial rate of cellulase production was slightly greater without the addition of oxygen

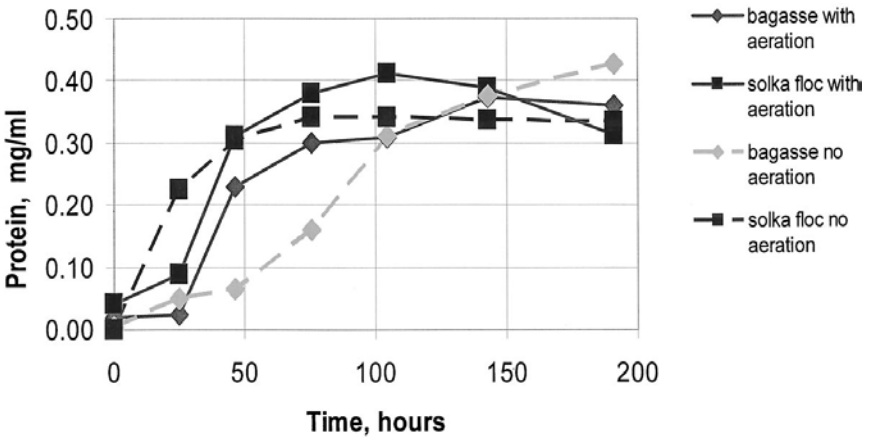

Fig. 10. The effect of aeration on protein production with Solka Floc and pretreated bagasse solids.

for the Solka Floc control,productivity was greater with the addition of oxygen for pretreated bagasse, but the final enzyme production titer was greater without oxygen in this case. These results suggest that enzyme production is not limited by oxygen concentrations at 10 g/L of cellulose for Solka Floc. However, the results also indicate that oxygen may be limiting at the higher solids levels of the pretreated materials or that aeration may remove some inhibitors such as dissolved lignin decomposition products and reduce the lag time for pretreated biomass. Removal of inhibitors appears consistent with other observations noted, but time was inadequate to perform additional experiments and conclusively resolve which of these possibilities was responsible for these results.

Discussion

Cellulase was produced on liquid hydrolysate alone and residual solids alone for bagasse pretreated with hot water in a flow system. The liquid fraction was found to be inhibitory to protein production and organism growth. Although we could adapt Rut C30 to improve its tolerance to the hydrolysate, this trait was not preserved in cultures grown from spores from the adapted strains, limiting the utility of this method. We also found that we could reduce the inhibitory effects of the hydrolysate by filtering it either before or instead of autoclaving.

Cellulase production was also found to be delayed for the pretreated solids, even after significant washing, and a 1- to 2-d lag was observed before protein production began. However, once initiated, the rate of protein release was very high, equaling or exceeding that for controls grown on Solka Floc, provided the solids were extensively washed. Cellulose levels and solids concentrations did not significantly impact cellulase productivity. However, adding lignin to Solka Floc or glucose resulted in a lag in

enzyme production similar to that observed for pretreated solids, and aerating the fermentations appeared to reduce the lag time.

More work is needed to pinpoint what caused the lag in enzyme production on pretreated solids. First, we need to find out whether this delay could simply be the result of enzyme adsorption on the greater amounts of lignin and cellulose, thereby slowing the initial growth of the organism. On the other hand, the delay in growth on glucose suggests that inhibitors cause this delay; the challenge would then be to develop approaches to either remove the inhibitors or adapt the organism to their effects. In this case, other strains of cellulase producers should also be evaluated to determine whether they are more tolerant to inhibitors. Continuous cellulase production could be particularly effective because the organism will acclimate and the startup time responsible for a lag in performance may be eliminated, although other results have found lower productivity for continuous vs fed-batch production *(6)*. Additionally, it would be valuable to determine how inhibition on materials from differential flow pretreatment with hot water compares with that for conventional dilute-acid and other pretreatment technologies because it well may be that materials from the latter systems impact cellulase production even more.

Acknowledgments

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