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Recycle of the Cellulase–Enzyme Complex After Hydrolysis of Steam-Exploded Wood

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ABSTRACT

Cellulases can be recovered in high yields by contacting fresh substrate with hydrolysis filtrate and by extraction of spent hydrolysis residue with pH 7 buffer. Recycled enzymes give hydrolysis rates about equal to those with fresh enzymes. Steam-exploded wood (SEW) is washed with water to remove sugars and byproducts from breakdown of hemicellulose, and recycle of enzymes proceeds better if lignin is also removed prior to hydrolysis. Oven drying of SEW interferes with recycle, and the recovery of enzymes is only one-half of that with SEW that is kept moist.

Effectiveness of enzyme recovery depends on the completeness of hydrolysis, as determined by contact time and enzyme concentration. For cost-effective operation, enzyme should not be recovered until appreciable filter paper activity and carboxylmethylcellulase activity appear in the hydrolysate.

Index Entries: Cellulase–enzyme complex, recycle of; steam-exploded wood, hydrolysis of; cellulase; lignin; hemicellulose; glucose; enzyme production; fuel-grade alcohol.

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INTRODUCTION

Glucose from enzymatic hydrolysis of wood can be fermented to fuel-grade alcohol, but the costs for producing enzymes are excessive. A good method of recovering enzymes for recyle to fresh substrate could be a key economic factor. We have demonstrated the release of cellulase enzymes from the hydrolytic residue of steam-exploded wood (SEW) by pH manipulation (*1*). The desorbed enzymes can be resorbed to fresh SEW for additional cellulolysis. This process was examined further to determine the technical feasibility of recovery of enzymes in large-scale systems.

Suspensions of SEW in acetate buffer were treated with cellulases. In commercial processes, much higher concentrations will be used (*2*), but the kinetics of sorption–desorption are less clear in these concentrated suspensions. In preliminary tests to establish the time course of the production of reducing sugar, filter paper (FP) activity was monitored to ascertain the distribution of activity between the residue and the hydrolysis fluid.

The binding strength of the enzyme–substrate complex is determined by the chemical environment; thus, the desorption of enzyme is easiest when the binding strength is minimum. From a practical viewpoint, however, the timing for enzyme recovery is determined by the diminishing rate of glucose production and the distribution of the components of the enzyme system between residue and supernatant.

The IOTECH steam-explosion procedure disrupts the structure of wood to expose cellulose fibers, and lignins are present in the SEW product. Nonspecific sorption of enzymes to cellulose, as well as to lignin, will occur. The IOTECH product was treated to remove lignin prior to cellulolysis to determine the effects on enzyme recovery and glucose yield.

MATERIALS

Steam-exploded wood from mixed hardwood species was provided by the IOTECH corporation. Portions of the fibrous material were stored frozen. The material remained friable in the frozen state and was washed three times with tap water and three times with distilled water before use. With each washing, the fines were discarded. Drained samples were oven-dried at 55°C, but drying was found to impair recycle of the enzymes. Emphasis then shifted to washed wood without drying.

Steam-exploded wood that had been washed with water was delignified by treatment with 1.0*M* NaOH. Washing removed 24% of the initial weight (hemicellulose). Alkali removed an additional 30% of the initial weight (lignin). This delignified SEW was also stored frozen. Thawed in distilled water, the material was washed, drained, and oven-

dried at 55°C, but eventually drying was omitted because of its undesired consequences.

Enzyme from Dr. Bland Montenecourt, Lehigh University, was frozen *Trichoderma reeseii* P-37 filtrate. The material was thawed, subdivided into vials after thorough mixing, and refrozen. Thawed samples were mixed before withdrawing aliquots because the enzyme system has a tendency to leave solution and form fine particles. The enzyme preparation contained 3.3 mg/mL folin protein (3). Replicate assays showed a range of 7–12 filter-paper units (FPU)/mL; 0.37 U/mL enzyme solution produces 2.0 mg reducing sugar, as glucose, from Whatman No. 1 paper, at pH 4.8, at 50°C. Beta-glucosidase (BG) was #G8625, Type II (Sigma Chemical Co., St. Louis, MO), 5.8 units/mg; 1 U produces 1.0 mol glucose from salicin/min, at pH 5.0, at 37°C. All incubations were at 45–50°C.

METHODS

Glucose was assayed by a modification of the glucose–oxidase–peroxidase method (4), utilizing Statzyme enzyme reagent purchased from Worthington Diagnostics Systems, Inc. Freehold, NJ.

Reducing sugars were measured by the dinitrosalicylic acid (DNS) procedure (5), in which 3.0 mL DNS reagent is added to a 1.0 mL sample in a boiling water bath for 5 min. After cooling to room temperature, transmittance is read vs a water blank after correcting with a reagent blank.

Overall activity of the cellulase–enzyme complex was measured as FP activity, according to the US Army Natick procedure. Filter-paper units were calculated from reducing sugar formed in 60 min. A 0.5 mL enzyme sample in citrate buffer for 1 h, with a 1 × 6 cm strip (50 mg) of Whatman No. 1 FP, gave additional reducing sugars, as determined by the DNS method.

Carboxymethyl cellulase (CMC) activity measures endoglucanase activity. The US Army Natick method uses 2.0% CMC (Hercules 7L2P; 70% substituted, low viscosity). A sample of enzyme is incubated with CMC, and the additional reducing sugars are assayed by DNS. The calculation is based on production of 1.0 mg of reducing sugar, as "glucose," in 30 min by a 0.5 mL sample (0.185 IU).

Beta-glucosidase activity was measured by the US Army Natick method with glucose determination, using Statzyme enzyme reagent (Worthington), following hydrolysis of 15 m*M* cellobiose for 30 min. One milligram of glucose represents 0.185 IU of cellobiase (beta-glucosidase) activity at critical dilution.

The assays used in this study (except for protein and glucose) all utilize the DNS measurement as part of the assay procedure. Therefore, a correction is made in the enzyme assays for reducing sugar in the wood hydrolysate sample. Blank contributions by residual reducing sugar in

the washed wood and by the DNS reagent blank are subtracted in the calculation of both enzyme activities and SEW hydrolysis rate.

Two major sources of error in the data are variations in the stock enzyme suspension and error magnification in dilution calculations. Since the method is very sensitive, extensive dilution is needed to bring readings into the proper range. Glucose and BG measurements have fewer calibration uncertainties because glucose standards are used for the end product, which is glucose. In addition, readings are made against a reagent blank rather than a water blank.

Hydrolysis in 125 mL Erlenmeyer flasks used 3.0% SEW, buffer, and enzyme at 45°C. The flasks were stoppered to prevent evaporation. Based on FPU, the specific activity of the P-37 enzyme stock was 7–12 U/3.3 mg or 2.1–3.6 U/mg protein. Because of this variation of the stock enzyme, each flask was assayed for enzyme concentration before adding SEW. After the addition of the wood, samples were taken at intervals from the supernatant of the flasks and assayed for sugars and enzymes.

Recycling of enzymes is illustrated in Fig. 1. Wood residue is separated from the supernatant and diluted with pH 7.0 phosphate buffer. At first, the flask contents were poured into cheesecloth and squeezed to get liquid for assays. The residue was resuspended in pH 7.0 buffer for 1 h, and the separation was repeated. This supernatant was adjusted to pH 4.5, assayed, and used to suspend fresh SEW for a second hydrolysis. Passing the hydrolysate through fresh SEW completes the process of enzyme recovery.

Another recycle procedure for experiments with fresh SEW passed buffer semicontinuously through a column packed with SEW and maintained at 45°C. Buffer (pH 4.5), enzyme, and 3% drained, but not dried, SEW were placed in a flask; after 1 h, the suspension was poured into a glass column containing a sintered-glass support. After 24 h, the buffer was drained from the column and assayed for enzyme and sugar. The buffer was replaced with pH 7.0 phosphate buffer that was allowed to drain through the wood residue. The pH of the eluate was adjusted to pH 4.5 and assayed for enzyme and sugar. Fresh SEW was mixed with the eluate and placed in another column to repeat the process.

Three standard curves were required for the DNS method because conditions of the measurements differ with regard to sample size and viscosity. At both lower and higher concentrations, the curves depart from linearity. Total reducing-sugar determinations were based on glucose standards.

RESULTS AND DISCUSSION

Figure 2 is an 18-d record of hydrolysis with 0.3 and 3.0 FPU in a 3% suspension of washed and dried SEW. The higher concentration of enzyme shows rapid hydrolysis with little change after 4 d. The lower con-

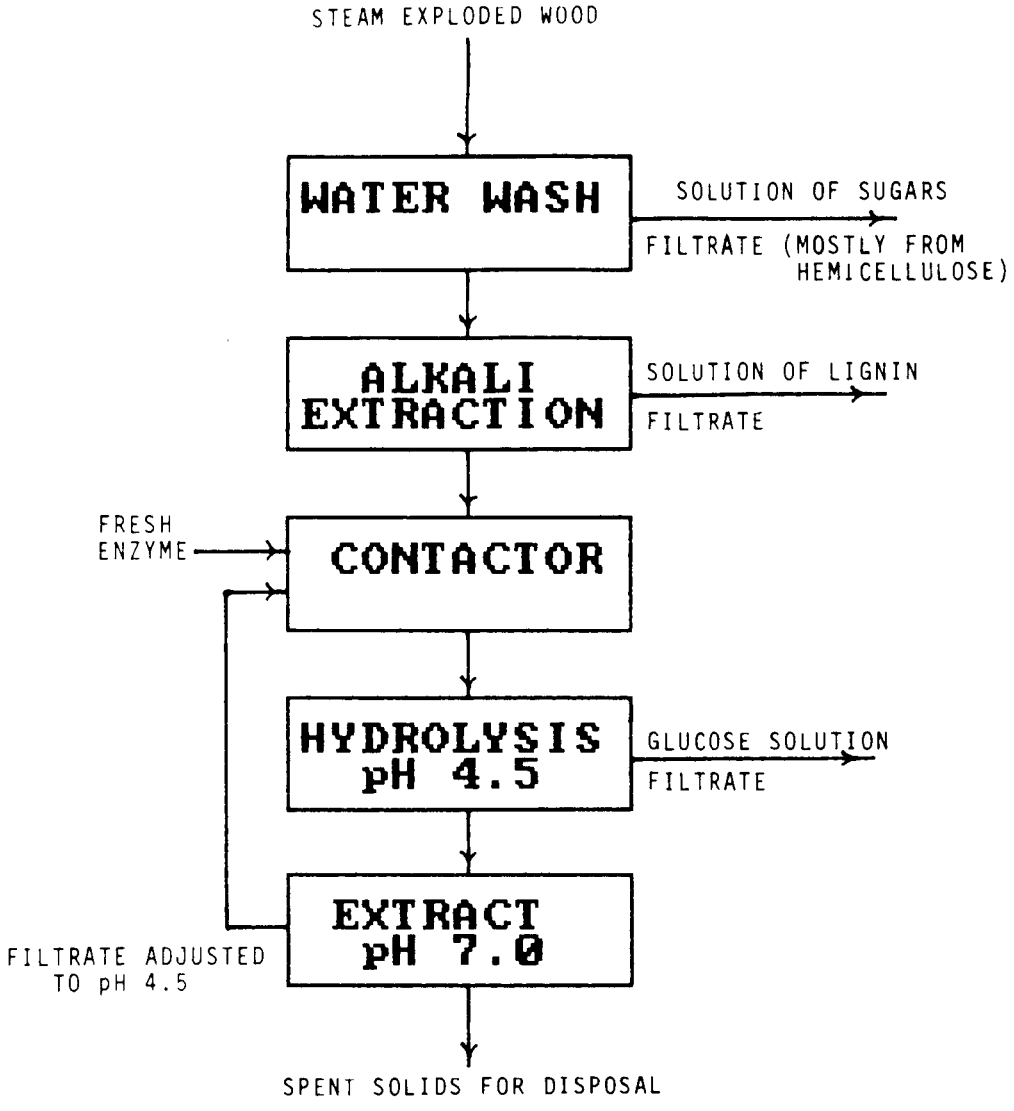

Fig. 1. Partial flowsheet for process.

centration gives a poor yield even at extended time and should not be considered for a practical process. There is also a high probability of contamination if this mixture of sugars and proteins is held for prolonged periods.

As reported in the literature (*6*), a 10-fold increase in enzyme may only double the rate of sugar production. This could be explained by nonspecific binding of enzyme to SEW. Early in hydrolysis, our system produced five times the product, with 10 times the enzyme, which is closer to the expectation for an enzyme-saturated system. It would be of interest to explore those properties of SEW that influence hydrolysis kinetics.

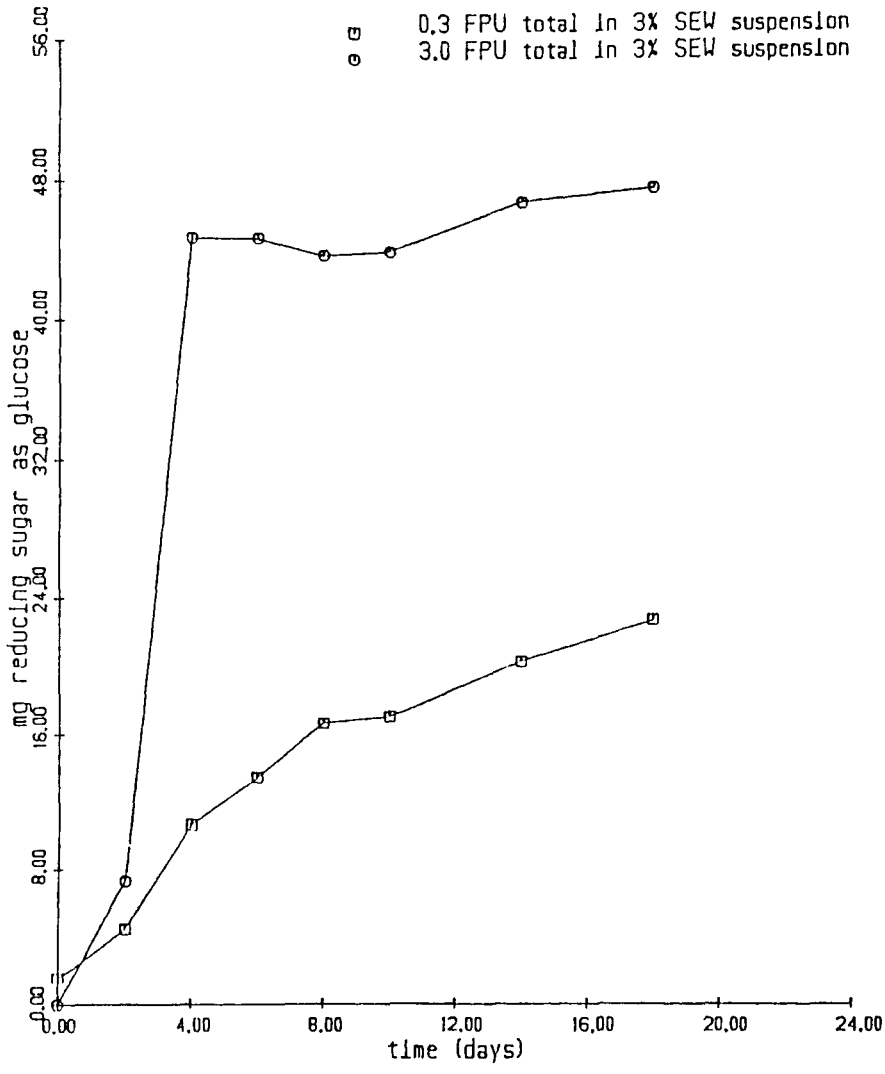

Fig. 2. Time course of hydrolysis.

Table 1 shows some characteristics of a system after hydrolysis. The initial FP activity averaged 0.48 FPU/100 mL. Carboxymethyl cellulase activity seems to be very labile because it dropped rapidly from its 2-h value of 7.6 CMC U/100 mL. The yield of glucose appeared low compared to the total reducing sugar, therefore, the run was repeated with additional BG. Results in Fig. 3 demonstrate possible deficiency of BG in the P-37 preparation. Subsequent experiments had additional BG (44 U/100 mL flask) to insure that the systems were not BG limited.

Several experiments using the protocol illustrated in Fig. 1 gave the results shown in Table 2. When SEW had been reacted from 2–4 d with enzyme (0.5 FPU/100 mL), the solids were separated and the residue eluted with pH 7.0 buffer. The supernatant and eluate were assayed to determine the degree of recovery and distribution of FP activity, as a

TABLE 1
Final Conditions of SEW Hydrolysate

Factor	Final assay, mg or units/100 mL
Total reducing sugars	68.0 (mg)
Glucose	16.7 (mg)
Filter paper units	0.2
Carboxymethyl cellulase units	0.0
Beta-glucosidase units	2.0

[a]Total hydrolysis time = 8 d.

function of time of contact with SEW. The table shows that enzyme is more readily recovered when the wood has been reacted with enzyme for more than 2 d under enzyme-saturated conditions. The 4-d system showed greater than 100% of the original activity, but this may be explained by imprecision of the methods. The high-sugar background produced by hydrolysis of SEW is a source of error.

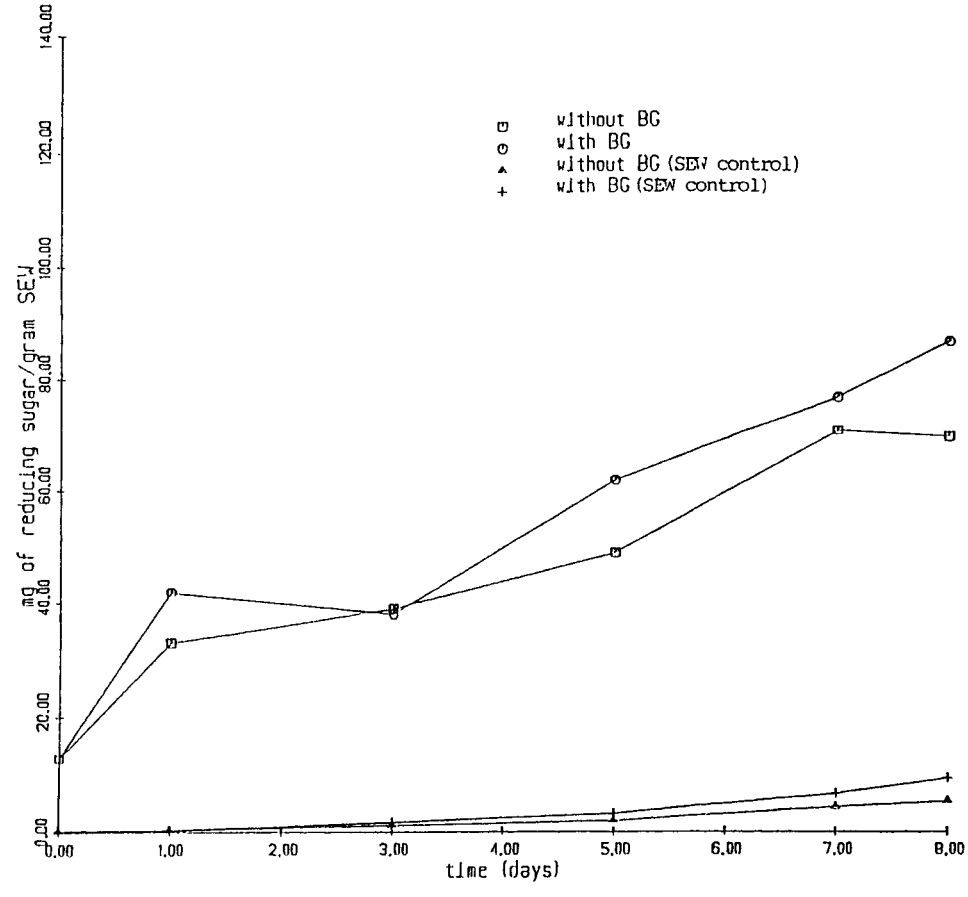

Fig. 3. Effect of additional beta-glucosidase.

TABLE 2
Enzyme Recovery for 2- and 4-d Hydrolysis

Time, d	% In supernatant	% In desorption buffer	Total
2	37	25	62
4	>100	50	>100

Table 3, which is for a similar experiment, but with 10 times the enzyme concentration, shows that for an equivalent dry weight of wood, enzyme recycles better (based on FP activity) from delignified wood than nondelignified wood. The lower recovery in SEW may be a result of nonspecific binding of enzyme to lignin.

Comparison of Tables 2 and 3 shows that for the same period (2 d, and hydrolysis incomplete) the percentage recoveries of enzyme from both the supernatant and the wood desorption eluate are different. This difference has been seen several times and most likely is a direct result of the enzyme concentrations used in these experiments. For any given enzyme concentration, distribution and recovery are dependent on time of hydrolysis. As hydrolysis goes to completion, appearance of enzyme in the supernatant and ease of desorption from SEW increase. There may be an economic tradeoff in a slight sacrifice in yield if enzyme recovery is improved.

It is probable that commercial cellulose processes will operate with high solids and 5.0–7.0 FPU/mL (2). As solids dissolve, more will be added. A limiting factor will be the ability to mix the suspension with affordable agitators. This commercial process may be an enzyme unsaturated system that optimizes for productivity, but not enzymatic efficiency. Under these enzyme-unsaturated conditions, enzyme may reside in the aqueous phase, thus increasing the FP activity of the supernatant.

Table 4 illustrates total reducing sugar from SEW and delignified SEW at two enzyme concentrations, with a recycle of the lower-concentration enzyme system at 24 h. A nearly sevenfold increase in sugar production is achieved with this concentration, using delignified SEW. Sugar productivity before and after enzyme recycle is another measure of ability to desorb enzyme from spent SEW. Enzyme recycle at

TABLE 3
Enzyme Recovery from SEW and Delignified SEW after 48 h of Hydrolysis

Substrate	% In supernatant	% In desorption buffer	Total
SEW	29	12	41
Delignified SEW	40	20	60

TABLE 4
Total Reducing-Sugar Production from SEW and Delignified SEW vs Enzyme Concentration and Recycle

System	Time, d	SEW, mg/L	Delignified SEW, mg/L
1× Enzyme	0	0	0
	1	257	200
	2	144	108
10× Enzyme	0	0	0
	1	1170	1350

d 1 in this case results in a 50% return of productivity. In previous studies at this initial concentration, peak productivity was not reached until much later than 24 h. This may account for the ease of desorption in these systems. At the higher (10-fold) enzyme concentration, productivity is much greater at 24 h than in the lower-concentration system. Recycle was not attempted for the higher-concentration system.

Simple pH adjustment of the wood hydrolysate effectively elutes the FP activity from the wood. Electrostatic forces may be important, and at neutral pH, the enzyme is more negatively charged. Since the overall charge of the polysaccharide and phenolic groups on the wood is also negative, the binding (especially nonspecific) will be weaker at neutral pH. Table 5 shows the effect of simple pH adjustments on SEW and delignified SEW for a 10-fold enzyme system.

This experiment demonstrates several of the general trends, such as greater enzyme elution into the supernatant from the delignified SEW than from SEW with lignin. Again, the appearance of enzyme in the supernatant and ease of elution into the supernatant correlates with good yield of hydrolysis (d 3). Since this procedure monitors the effect of pH manipulation without recycling enzyme onto new SEW, another set of recycle experiments was performed. Dried SEW had been used for all of the above experiments. In the final group of experiments, wood that had

TABLE 5
Enzyme Elution by pH Adjustment

Substrate	Time, d	% FPU at pH 4.5	% Eluted at pH 7.0
SEW	1	11.4	100
	2	16.2	23.1
	3	47.4	57.8
Delignified SEW	1	43.5	100
	2	20.5	100
	3	92.3	100

not been dried was added to a buffered enzyme solution (10-fold). The results of a 2-d recycle of enzyme from wet wood products (SEW, delignified SEW, and 50.0% delignified SEW), following the procedure of Fig. 1, show that almost all of the enzyme can be desorbed from wet SEW. The difference observed between this experiment and that reported in Table 2 shows that enzyme is more readily desorbed from wet wood than from dry wood at 2 d of hydrolysis. The dry system resembles the wet system after 4 d.

Preliminary experiments with dry SEW have shown that the spent hydrolysate, when recontacted with fresh SEW, also produces more reducing sugar. As much as 70.0% of FPU and CMC activities (assayed in the supernatant just before recycle) can be recycled in a system with dried SEW when hydrolysis has gone to completion. To insure that this process does not sorb sugars from the hydrolysate onto the fresh wood, samples of SEW hydrolysate were assayed for total reducing sugars before and after contact with fresh SEW. The per cent transmittance of the effluent after an hour of contact with the new wood was unchanged, indicating that sugar content in the hydrolysate was the same as before contact with new SEW.

CONCLUSION

This study suggests the following: (1) Enzyme recovery is dependent upon time of hydrolysis and initial enzyme concentration. At high concentration greater than, etc. (>0.6 FPU/100 mL), enzyme recycles more readily from SEW residue and supernatant when reacted for more than 48 h on both lignified and delignified SEW; (2) enzyme recycles better from wet SEW than from dried SEW, and (3) at high enzyme concentrations, all of the cellulose hydrolyzing activity can be recycled by washing the 48-h residue with pH 7.0 elution buffer and passing the eluate plus the hydrolysate through new SEW. This treatment does not retain sugars from the hydrolysate.

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