0273-2289/99/77–79/0435/$12.50

Bioconversion of Secondary Fiber Fines to Ethanol Using Counter-Current Enzymatic Saccharification and Co-Fermentation

Thomas W. Jeffries* and Richard Schartman

USDA, Forest Service, Forest Products Laboratory,
One Gifford Pinchot Drive, Madison, WI 53705,
E-mail: twjeffri@facstaff.wisc.edu

Abstract

This research examined several enzymatic and microbial process for the conversion of waste cellulosic fibers into ethanol. The first was a one-stage process in which pulp fines were contacted with commercial enzyme solutions. The second process used sequential, multistage saccharification. The third used sequential enzyme addition in a countercurrent mode. Experiments compared the results with various feedstocks, different commercial enzymes, supplementation with β-glucosidase, and saccharification combined with fermentation. The highest saccharification (65%) from a 4% consistency pulp and the highest sugar concentration (5.4%) from an 8% consistency pulp were attained when 5 FPU/g plus 10 IU/g of β-glucosidase were used. Sequential addition of enzyme to the pulp in small aliquots produced a higher overall sugar yield/U enzyme than the addition of the same total amount of enzyme in a single dose. In the saccharification and fermentation experiments, we produced 2.12% ethanol from a 5.4% sugar solution. This represents 78% of the theoretical maximum. This yield could probably be increased through optimization of the fermentation step. Even when little saccharification occurred, the enzyme facilitated separation of water, fiber, and ash, so cellulase treatment could be an effective means for dewatering pulp sludges.

Index Entries: Cellulase; secondary fiber fines; bioconversion.

Introduction

Paper recycling can reduce timber demand and save landfill space. However, not all recycled paper ends up on the consumer's shelf. Papermaking invariably damages and shortens pulp fibers, and a fraction of the feedstock—approximately 15–20%—is removed as fines along with clays and fillers *(1,2)*. The most cost-effective means of dealing with this fraction

*Author to whom all correspondence and reprint requests should be addressed.

is to landfill it. Landfill costs vary regionally. Recycle mills are often located close to municipalities to reduce collection and distribution costs, but in these same regions, landfill costs can be high. The sludge, moreover, is wet and heavy, so it is expensive to transport, and once it is placed in a landfill, it is anaerobically digested, and can cause excess acid accumulation and seepage of organic materials into the soil or run-off. These problems are especially prevalent if the landfill is not designed to accept wet sludge. Other disposal methods include land spreading and combustion *(3)*. Combustion is not usually feasible because of the high water and clay content. Paper recycling mills are trying to minimize byproduct and effluent production and maximize co-products. This is especially important to companies that emphasize green marketing. So, for recycling programs to work well, cost-effective solutions are needed for sludge disposal.

Cellulosic fiber fines from paper recycling mills are potential feedstocks for ethanol production *(4)*. Their characteristics vary widely, and they can contain various contaminants, but some mills use only chemical fibers. These feedstocks consist mostly of kraft-pulped, bleached cellulose that is essentially free of lignin. Approximately 15% of the incoming waste paper cannot be recycled into paper products. With an increase in recycling rates, disposal of this fraction is becoming more difficult.

We evaluated the potential for enzymatic saccharification and fermentation to convert recycled fiber fines into ethanol *(5)*. The fines were taken from a pulp mill that used only bleached chemical pulp (office waste paper) as a feedstock, so lignin inhibition wa s not a problem. Also, because we worked only with the fiber fines, particle size was not a difficulty. The approach consisted of enzymatic degradation of cellulosic fines with subsequent fermentation using yeasts. Inks and additives in the sludge (biosurfactants and antimicrobials to block pulp degradation) did not present problems. Alkaline fillers present in the recycled fiber caused difficulties. Saccharification followed by fermentation was technically feasible. Potential improvements include the staged addition of enzyme, and the use of a countercurrent reactor.

Materials and Methods

Three samples of recycled fiber fines were supplied by EcoFibre, Inc., a subsidiary of Riverside Paper Company (De Pere, WI). These were analyzed gravimetrically for water and ash content. After drying, the amount of lignin and the sugar composition was determined by acid hydrolysis and ion chromatography with pulsed amperometric detection of individual sugars *(6)*. Pulp fines contained 22–33% ash and 45–65% fermentable carbohydrates. Saccharification was carried out with commercial *Trichoderma reesei* cellulase complexes for 12–48 h. The pH was adjusted to 4.5. Two pulp consistencies (4% and 8%), and two enzyme-loading rates (1.5 and 5.0 filter paper U/g) were employed in initial batch studies. Total loading rates were higher in sequential or countercurrent batch.

Four commercially available enzyme preparations were characterized for catalytic activity. Cellulase was measured by exposing Whatman #1 filter paper to enzyme and monitoring the rate of saccharification by the dinitrosalycilic (DNS) acid method *(7)*. β-glucosidase was analyzed in a similar manner except the substrate was 2-(hydroxymethyl) phenyl glucopyranoside. All enzyme activities were expressed in terms of international units (IU, μmoles of sugar produced/min). Saccharification was characterized by measuring pulp mass prior to and after digestion and by the DNS method for reducing sugars.

Saccharification was carried out at 50°C in citrate buffer solutions (0.05–0.1 *M*, pH 4.8). The consistencies (solids contents) of reaction mixtures were adjusted to various levels as given in the text. Consistency was calculated and is expressed in terms of carbohydrate mass rather than total dry mass. The ash contents were a significant fraction of the total dry weight, and they varied from one batch to another. The as-received cellulosic fines contained a component—presumably calcium carbonate—that rendered the digestion mixture basic. Therefore, the pulp was neutralized with sulfuric acid prior to the addition of enzymes and buffer. A countercurrent hydrolysis was mimicked by use of three batch reactions (Fig. 1). The saccharification steps illustrated in the figure were performed at 12-h intervals and control samples without enzyme additions were run alongside the samples being tested. The experiment was run several times to investigate the effects of enzyme dose and consistency, but the results from only one trial is presented here.

A hydrolyzate produced from waste pulp was fermented with four different yeasts: *Candida shehatae* ATCC 22984, *Pichia stipitis* CBS 6054, *Saccharomyces cerevisiae* NRRL Y-2034, and *S. cerevisiae* ATCC 26785. To prepare a hydrolyzate for fermentation, we used 8% consistency of batch 1 fines, 5.0 IU/gram carbohydrate of Multifect CL and 10 IU/gram carbohydrate of Novozym 188 β-glucosidase. The resultant sugar concentration in the supernatant solution was 5.4% by mass. Fermentation was carried out in a shake flask at 30°C and 100 rpm. Ethanol production was monitored by gas chromatography (GC) as previously described *(8)*.

Results and Discussion

Assays

Three separate samples of fiber fines were obtained and analyzed. Their moisture contents were 58.9, 5.4, and 67.8 percent by mass, for batches 1, 2, and 3, respectively. The second batch of fines was dried prior to shipping and was very stable during storage. Batches 1 and 3 were not dried, and within a few weeks anaerobic acidogenic fermentations had occurred even though all three pulps were held in cold storage (3°C). Batches 1 and 2 came from early runs at the plant and had relatively low ash contents. As the fiber-recovery processes in the plant improved, more of the fiber was recovered. Sample 3 had the highest ash content, the least carbohy-

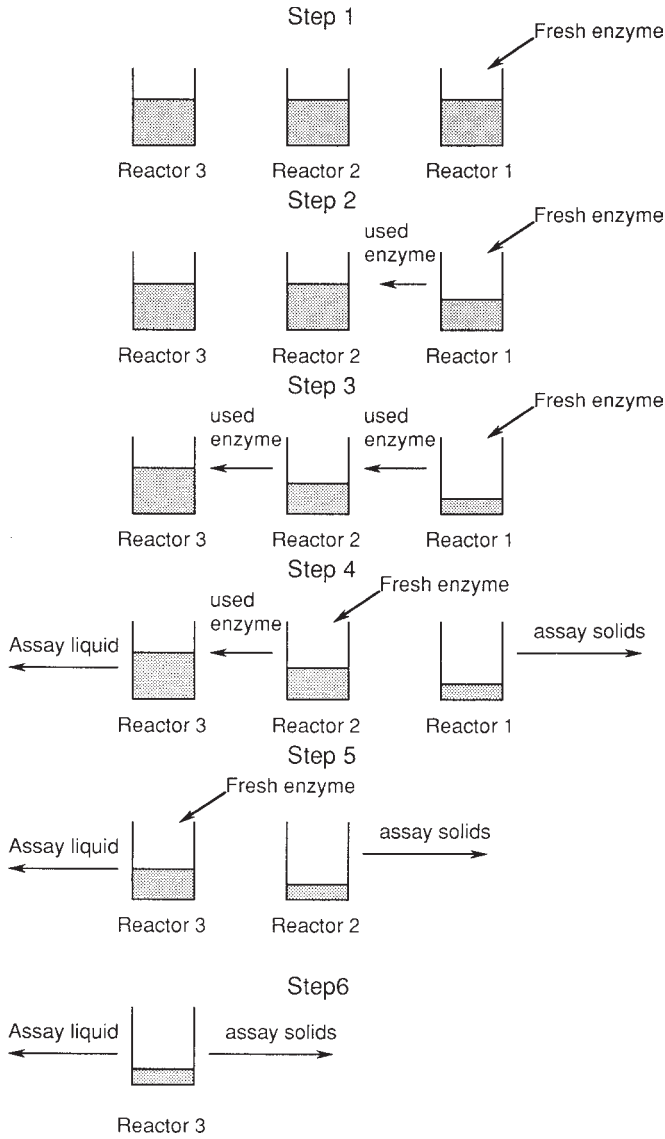

Fig. 1. Scheme for multi-stage countercurrent batch saccharification of cellulosic fiber fines. Results are found in Table 8.

drate, and the highest Klason lignin content. In all cases, the dominant sugars were glucan and xylan. The results of the analysis of the three cellulosic fiber samples are given in Table 1.

The filter paper assays for cellulase activity showed the following results: Celluclast™ 1.5 L, 98 IU/mL; Cytolase™ CL, 67 IU/mL; Novozym™ 188, 5.2 IU/mL, and Multifect™ CL, 93 IU/mL. The cellobiase assay of Novozym 188 showed a cellobiase activity of 250 IU/mL. Enzyme activities can vary from batch to batch, and the methods for manufacturing these

Table 1
Composition of the Dry Matter in Three Batches of Cellulosic Fiber Fines

Sample	Ash[a] %	ASL[b] %	KL[b] %	Arab[b] %	Galac[b] %	Gluc[b] %	Xyl[b] %	Man[b] %
1	21.8	0.81	18.1	.1	.1	56.0	12.5	1.9
2	21.8	1.40	20.7	.2	.2	48.8	11.8	1.5
3	32.6	1.10	25.3	.1	.1	38.5	9.0	1.2

[a]Expressed as a percent of the total dry matter.
[b]Expressed as a percent of the total organic matter.
KL, Klason lignin; Arab, arabinose; Galac, galactose; Gluc, glucose; Xyl, xylose; Man, mannose.

Table 2
Gravimetric Analysis of Saccharification Following Enzymatic Hydrolysis

Consistency %	Enzyme dose[a] %	Batch 1 %	Batch 2 %	Batch 3 %
4.0	1.5	34.2	20.9	19.9
4.0	5.0	44.2	46.9	37.5
8.0	1.5	15.3	15.9	13.3
8.0	5.0	31.5	32.8	25.9

[a]IU/g carbohydrate.

commercial preparations change, so these results should not be taken as a measure of enzyme quality when selecting a manufacturer.*

Feedstock and Enzyme Comparisons

We compared Celluclast, Cytolase, and Multifect enzymes for their relative abilities to hydrolyze batch 1 as measured by the DNS method. Hydrolysis ceased after 40 h. No additional sugar release was observed after extending the experiment out to 1 wk (data not shown). The amount of sugar produced did not scale with the enzyme dose. When the three commercial enzyme preparations were applied at the same filter-paper activities, they gave similar degrees of hydrolysis.

The three different cellulosic feedstocks were compared for their ease of hydrolysis as measured by the gravimetric assay. Saccharifications were carried out at 4% and 8% consistency along with enzyme doses of 1.5 and 5 IU/g carbohydrate for 37.5 h (Table 2). Samples 1 and 2 gave similar results except at the lowest dose and consistency. However, significantly more sugar was released from batch 1. This could have been attributable to the inefficiency of fiber removal in the early stages of the plant operation. Batch 1 also had the highest glucan and xylan contents. Batch 3 gave con-

*Trade names are offered for the convenience of thc reader and do not represent an endorsement by the USDA, FS, or the superiority of one product over another.

sistently lower levels of saccharification than batches 1 and 2. As noted previously, it also had the highest ash content.

Multistage Hydrolysis

Higher degrees of saccharification and higher sugar concentrations are preferred for commercial purposes than seen in our previous experiments. At the same time, the enzyme dose must be minimized. Because product inhibition can significantly impede enzyme activity, we investigated whether hydrolysis of samples would be more effective with the periodic addition of smaller amounts of enzyme. We carried out saccharification on solkafloc samples and on sample 1 using two consistencies and two dosage levels. We did not keep the consistency and enzyme dose constant from one stage to the next, because exact control of these quantities would require knowledge of the degree of saccharification at each stage of enzyme addition. The gravimetric and DNS assays do not agree, which makes control of these factors imprecise.

The extent of hydrolysis decreased with increasing consistency. At the lowest level, lowest dosing rate, and the highest consistency, as little as 13.3% of the carbohydrate was solubilized. At the lowest consistency and highest dosing rate, 46.9% of the carbohydrate was solubilized. The apparent amount of sugars solubilized as measured by the DNS method was considerably lower than that measured gravimetrically.

Even though relatively little solubilization occurred at the lowest dosing rates, cellulase had a profound effect on water separation and sludge filterability. Within 12–24 h, the suspensions were noticeably less viscous and after 37 h, they had separated into distinct aqueous and solids layers. Control pulp sludges showed no such change. These effects depended on the enzyme dose. Cellulase treatment could, therefore, be an effective means for dewatering pulp sludges.

The consistencies and enzyme dose in each stage of hydrolysis after correcting and calculating for residual solids are shown in Table 3. The raw results of the sugar assays after each stage of the experiment are displayed in Tables 4–6. In the first 12 h, sugar release was greater at higher consistency and with larger enzyme doses. Generally, the gravimetric determination indicated higher levels of solubilization, but in a few instances, the DNS assay indicated greater sugar production With the second stage of enzyme addition, sugar release increased. This was particularly true in those samples in which enzyme had been limiting in the first stage addition. A minimal amount of enzyme was necessary for sugar release or solubilization.

Nothing present in the sludges prevented saccharification, because in most instances the substrate saccharified as well or better than the solkafloc controls. Enzyme addition in stages resulted in more saccharification than the application of an equivalent amount of enzyme in one large dose. Differences were evident in the results of the two methods used to assay

Table 3
Enzyme Dosing Rates in Multi-Stage Saccharification
as Determined by Gravimetric Analysis of Residual Solids

	Stage 1		Stage 2		Stage 3	
Sample[a]	Consistency (% carb.)	Dose (IU/g)	Consistency (% carb.)	Dose (IU/g)	Consistency (% carb.)	Dose (IU/g)
S-4-1.5	4	1.5	4.0	1.50	3.8	1.5
S-4-5	4	5.0	4.0	5.00	3.8	5.0
S-8-1.5	8	1.5	8.0	1.50	6.8	1.5
S-8-5	8	5.0	8.0	5.00	6.8	5.0
R-4-1.5	4	1.5	5.4	1.15	3.6	1.5
R-4-5	4	5.0	5.8	3.36	3.6	5.0
R-8-1.5	8	1.5	10.0	1.22	6.3	1.5
R-8-5	8	5.0	11.2	3.67	6.0	5.0

[a]The first digit identifies the substrate: R, Riverside shipment 1 and S, Solkafloc control. The second parameter identifies the initial consistency and the third digit indicates the initial enzyme dose in IU/g carbohydrate.

Table 4
Results from Stage 1 Saccharification

Sample[a]	Initial carb. mass (g)	Saccharified carb. (grav.) (g)	Saccharified carb. (DNS) (g)
S-4-1.5	5	0.51	0.297
S-4-5	5	1.53	0.713
S-8-1.5	10	0.03	0.305
S-8-5	10	2.75	1.010
R-4- 1.5	5	0.46	0.541
R-4-5	5	1.62	0.872
R-8-1.5	10	0.00	0.811
R-8-5	10	1.67	1.490

[a]The first digit identifies the substrate: R, Riverside shipment 1 and S, Solkafloc control. The second parameter identifies the initial consistency and the third digit indicates the initial enzyme dose in IU/g carbohydrate.

saccharified carbohydrate. These were probably owing to the presence of carbohydrate that was not completely broken down to sugar monomers. Gravimetry measures residual undissolved carbohydrate, and oligomers up to 5 sugar units long are soluble. The DNS method measures the number of reducing groups formed when the carbohydrate chains have been hydrolytically cleaved. The difference in the two assays suggests that significant amounts of oligosaccharides were formed, and that supplementation of the cellobiase component would lead to higher fermentable sugar concentrations. When β-glucosidase was added to the hydrolysis mixtures, reducing sugar release increased by 18.3 and 14.9%, respectively (Table 7).

Table 5
Results from Stage 2 Saccharification

Sample[a]	Initial carb. mass (g)	Saccharified carb. (grav.) (g)	Saccharified carb. (DNS) (g)
S-4-1.5	3.94	0.56	0.37
S-4-5	2.84	1.13	0.41
S-8-1.5	9.02	1.09	0.46
S-8-5	6.45	1.75	0.58
R-4-1.5	4.05	0.76	0.30
R-4-5	2.85	0.82	0.27
R-8-1.5	9.18	2.41	0.59
R-8-5	7.44	2.26	0.68

[a]The first digit identifies the substrate: R, Riverside shipment 1 and S, Solkafloc control. The second parameter identifies the initial consistency and the third digit indicates the initial enzyme dose in IU/g carbohydrate.

Table 6
Results from Stage 3 Saccharification

Sample[a]	Initial carb. mass (g)	Saccharified carb. (grav.) (g)	Saccharified carb. (DNS) (g)
S-4-1.5	2.82	0.73	0.39
S-4-5	1.21	0.40	0.29
S-8-1.5	7.37	1.93	0.81
S-8-5	4.20	0.87	0.70
R-4-1.5	2.96	0.50	0.39
R-4-5	1.70	0.20	0.31
R-8-1.5	6.46	2.02	1.13
R-8-5	4.78	2.50	1.54

[a]The first digit identifies the substrate: R, Riverside shipment 1 and S, Solkafloc control. The second parameter identifies the initial consistency and the third digit indicates the initial enzyme dose in IU/g carbohydrate.

Table 7
Effect of β-glucosidase on Hydrolysis

Consistency (%)	Multifect CL dose (IU/gram carb.)	Novozym 188 dose (IU/gram carb.)	Saccharification (%)
4	5	0	44.2
4	5	10	65.2
8	5	0	32.9
8	5	10	47.8

Staged Countercurrent Hydrolysis

Because staged addition of enzyme was so effective, we decided to examine the effect of staged, countercurrent hydrolysis. In this process, the

Table 8
Saccharification in a Countercurrent Batch Process

Reactor	Consistency (%)	Dose (IU/g)	Saccharification (%)
1	4.0	1.5	38.9
2	4.2	1.5	10.4
3	4.3	1.5	10.6
Control	4.0	1.5	27.4
1	4.0	5.0	65.1
2	4.0	5.0	25.4
3	4.0	5.0	24.1
Control	4.0	5 0	57.7
1	8.0	1.5	31.1
2	8.0	1.5	5.2
3	8.0	1.5	4.9
Control	8.0	1.5	20.04
1	8.0	5.0	62.9
2	8.0	5.0	15.9
3	8.0	5.0	14.3
Control	8.0	5.0	43.4

supernatant solution from one hydrolysis was contacted with a second batch of fines, and fresh enzyme was added to the first batch. The process was continued until all three batches of fiber fines had been contacted with three fresh or supernatant enzyme solutions.

Gravimetric analyses of the pulp remaining after the end of the experiment showed that the pulp in reactors 2 and 3 had about the same degree of saccharification, whereas the pulp in reactor 1 had almost three times that level of saccharification (Table 8). The results indicate that most of the cellulase adheres to the fines in the first reactor.

The enzyme preparations have three enzyme components: β-glucosidase, endoglucanase, and exocellobiohydrolase. If the components become separated during the liquid-transfer process, then hydrolysis to glucose will cease. The results can be fully explained if the enzyme preparation has a highly soluble β-glucosidase component along with endoglucanase and exoglucanse components, which remain tightly bound to pulp. Upon transfer, the sugar concentration in the liquid does not increase, but the remaining endoglucanase and exoglucanse retained on the pulp will assist any fresh incoming enzyme with hydrolysis. In this way, a much higher degree of pulp saccharification was generated in reactor 1 as compared to reactors 2 and 3.

Fermentation

Fermentation was carried out as described in the Materials and Methods section. *P. stipitis* CBS 6054 gave the highest ethanol production at 2.1%, but yields from the other yeasts were similar. The yield of 2.1% represents

78% conversion of sugars dissolved in the hydrolyzate. As the sugar content is based on a thermogravimetric assay, some of the dissolved sugars may not have been broken down to monomer. Thus the yeast may simply have run out of fermentable material. Clearly, there is nothing inherent in the recycled pulp samples that prevents the use of biological techniques.

Conclusions

Ethanol production from waste pulp is technologically possible. The only modification needed was to neutralize a basic component of the pulp. The limitation to ethanol yields when using commercial enzyme preparations appears to be the stability of the β-glucosidase. This leads to sugar production that does not scale with enzyme dose and dissolved oligomers in addition to the monomer. These problems can partially be dealt with using staged enzyme addition or supplementation with enzyme having a strong β-glucosidase component.

Acknowledgment

The authors gratefully acknowledge the generous support of this research by the Riverside Paper Company in De Pere, WI.

References

1. McGovern, J. N., Berbee, J. G., Bockhelm, J.G., and Baker, A. J. (1983), *Tappi. J.* **66(3),** 115–118.
2. Joyce, T. W., Webb, A. A., and Dugal, H. S. (1979), *Tappi. J.* **62(4),** 83–84.
3. Diehn, K. and Zuercher, B. (1990), *Tappi. J.* **73(4),** 81–86.
4. Walkinshaw, J. W., Sladek, K. J., and Eberiel, D. T. (1984), *Tappi. J.* **67(10),** 104–105.
5. Wayman, M., Chen, S., and Doan, K. (1992), *Proc. Biochem.* **27,** 239–245.
6. Jeffries, T. W., Yang, V., and Davis, M. (1998), *Appl. Biochem. Biotechnol.* **70/72,** 257–265.
7. Miller, G. L. (1959), *Anal. Chem.* **31,** 426–429.
8. Jeffries, T. W. (1985), in *Energy Applications of Biomass*, Lowenstein, M. Z., ed., Elsevier, London and New York, pp. 231–251.