

# USING SIMONS STAIN TO PREDICT ENERGY SAVINGS DURING BIOMECHANICAL PULPING

*Masood Akhtar*

Research Scientist  
University of Wisconsin Biotechnology Center and  
the Institute for Microbial and Biochemical Technology  
USDA Forest Service, Forest Products Laboratory  
Madison, WI 53705-2398

*Robert A. Blanchette*

Professor

and

*Todd A. Burnes*

Research Scientist

Department of Plant Pathology  
University of Minnesota  
St. Paul, MN 55108

(Received August 1994)

## ABSTRACT

Pretreatment of wood chips with lignin-degrading fungi can save substantial amounts of electrical energy during a mechanical pulping process. In order to optimize this process, a rapid and reliable method was needed to predict energy savings. In this paper, we examine a fiber staining method that involves the use of Simons stain. This stain for microscopic examination of pulp fibers has been used previously to evaluate the degree of fibrillation in beaten fibers or more recently to differentiate fibers from untreated (control) and fungus-treated wood chips. Aspen or loblolly pine wood chips were treated with white-rot fungi under different experimental conditions in static-bed bioreactors for two or four weeks. At harvest, control and fungus-treated chips were refined through a single-disk mechanical refiner and then evaluated for fiber staining characteristics and the energy consumption during refining. Fibers obtained from control pulps stained a deep blue, whereas those obtained from different biopulps showed different intensities of yellow. The yellowing of biopulp fibers correlated very well with energy savings. The results demonstrate that the Simons staining method can accurately predict appreciable energy savings during biomechanical pulping and therefore can be used as a rapid screening technique to optimize the biopulping process.

*Keywords:* Simons stain, fiber analysis, fibrillation, mechanical pulping, biopulping, energy savings, white-rot fungi, *Phanerochaete chrysosporium*, *Ceriporiopsis subvermispota*.

## INTRODUCTION

Biomechanical pulping is the treatment of wood chips with white-rot fungi prior to mechanical pulping. At a laboratory scale, we have demonstrated the technical feasibility of this process; a pretreatment of both softwood and hardwood species with the lignin-degrading fungus *Ceriporiopsis subvermispota* prior to mechanical pulping reduced the electrical en-

ergy consumption, improved the paper strength properties, and reduced the environmental impact of pulping (Akhtar et al. 1992a, b, 1993; Akhtar 1994; Kirk et al. 1993; Sykes 1994). These results have demonstrated the benefits of biopulping; however, the process requires optimization, and follow-up engineering and scale-up studies before it becomes commercially available.

TABLE 1. Correlation of yellowing of fiber ends with that of energy savings using two different fungi on loblolly pine chips (2-week incubation).

Treatments	Blue	Yellowing of fiber ends		
		Slight	Inter	Advanced
Control	+			
<i>Phanerochaete chrysosporium</i> BKM F-1767	+ <sup>a</sup>	+ <sup>a</sup> (9%) <sup>b</sup>		
<i>Ceriporiopsis subvermispota</i> CZ-3			+	+ (16%)

<sup>a</sup> Two pluses for one treatment indicate that the staining pattern was in between the two categories.

<sup>b</sup> Values in parentheses represent energy savings compared to the untreated control.

Current methods for process optimization are very time-consuming and labor-intensive (Akhtar 1994; Blanchette et al. 1992b; Kirk et al. 1993). Therefore, a rapid and reliable screening method is needed. Unfortunately, during a previous investigation of biomechanical pulping, no relationship among wood biomass loss, the bulk removal of lignin, energy savings or strength, and optical properties was found (Leatham et al. 1990a, b). In another study, we found that PFI milling and freeness measurements can accurately predict paper strength (Leatham and Myers 1990). However, for energy savings, we concluded that the PFI milling method can be used only to discriminate fungal treatments from controls and not to discriminate among different fungal treatments (Akhtar et al. 1989).

Recently, a simple, but rapid screening method involving the use of Simons stain has been shown to have potential for evaluating biomechanical pulps (Blanchette et al. 1992a). This staining method has been used by others in various investigations to evaluate the degree of fibrillation in beaten fibers (Simons 1950; Wurz 1969). Simons stain is a differential stain made up of a mixture of a high molecular weight orange dye and a low molecular weight blue dye. Pulp absorbs the orange dye if the fibers are fibrillated or damaged, but stains blue when fibers are unchanged. Recently we established that Simons staining method can be used to discriminate fungal treatments from the controls for energy savings (Blanchette et al. 1992a). We further evaluated this approach to determine if this method can be used to monitor differences among fungal pretreatments. We have found that the intensity of yellow

staining of biopulp fibers correlates very well with energy savings.

## EXPERIMENTAL

### Fungal selection

Two fungi were used for this study: *Phanerochaete chrysosporium*, a partially selective lignin-degrading fungus, and *Ceriporiopsis subvermispota*, the most selective lignin-degrading fungus identified to date in our biopulping runs (Akhtar et al. 1993; Blanchette et al. 1992b). *P. chrysosporium* BKM-F-1767 and *C. subvermispota* CZ-3 (Table 1) were obtained from the Center for Forest Mycology Research (CFMR) of the Forest Products Laboratory (FPL), Madison, Wisconsin.

### Species and strains selection

Three different species of *Ceriporiopsis*—*C. rivulosa*, *C. pannocincta*, and *C. subvermispota*—and several strains of *C. subvermispota* were selected for use. The names of each species and strains are listed in Tables 2 and 3. These isolates were also obtained from the CFMR of FPL.

### Fungal optimization

In this series of experiments, CZ-3 strain of *C. subvermispota* was used. In one experiment, three different levels of liquid inoculum (0.01, 0.10, and 0.30% on a dry-weight basis) were used. In the second experiment, 0.10% inoculum (dry-weight basis) was used, but one bioreactor received a modified chemically defined medium (Leatham 1983), whereas the other received sterilized water only.

TABLE 2. Correlation of yellowing of fiber ends with that of energy savings using selected *Ceriporiopsis* spp. on aspen wood chips (2-week incubation).

Species/strains	Blue	Yellowing of fiber ends		
		Slight	Inter	Advanced
Control	+			
<i>Ceriporiopsis rivulosa</i> L-10602-sp	+ (0%)			
<i>Ceriporiopsis rivulosa</i> PiiRTO-26K225	+ <sup>a</sup>	+ <sup>a</sup> (3%) <sup>b</sup>		
<i>Ceriporiopsis pannocincta</i> FP-101181-sp	+	+ (4%)		
<i>Ceriporiopsis subvermispota</i> L-3292		+	+ (7%)	
<i>Ceriporiopsis subvermispota</i> CZ-3			+ (12%)	
<i>Ceriporiopsis subvermispota</i> L-9186-sp			+	+ (16%)

<sup>a</sup> Two pluses for one treatment indicate that the staining pattern was in between the two categories.

<sup>b</sup> Values in parentheses represent energy savings compared to the untreated control.

### Wood

Freshly cut loblolly pine (*Pinus taeda* L.) and aspen (*Populus tremuloides*) pulp wood-size logs were obtained from the Talladega National Forest in Talladega, Alabama, and the Nicolet National Forest, Wisconsin, respectively. Logs were debarked and chipped to a nominal 16-mm size. Chips were placed in plastic bags and frozen to prevent the growth of contaminating microorganisms.

#### *Inoculum preparation, chips preparation, and bioreactor inoculation*

The details about the inoculum preparation for each fungus, chips preparation, and bioreactor inoculation have been mentioned in our previous publications (Akhtar et al. 1992b; Fischer et al. 1994). The control and the inoculated bioreactors were incubated for 2 or 4 weeks at 27 C for *Ceriporiopsis* species/strains

and 39 C for *P. chrysosporium* and 65% relative humidity.

#### *Energy measurements*

At harvest, the untreated control chips and the fungus-treated chips were refined in a 300-mm-diameter disk refiner. After one pass through the refiner, a very small sample (but representative) of these coarse fibers from the control or the fungus-treated chips was then evaluated for Simons staining. The remaining samples were continued to be refined many times until the Canadian Standard Freeness (a measurement of water drainage) of the pulps dropped below 100 ml (Freenesses at different plate settings during refining were 649, 409, 234, 139, and 88 ml). Energy values for the control and the fungus-treated chips were regressed to 100 ml freeness for comparison. The details about the refining and the energy mea-

TABLE 3. Correlation of yellowing of fiber ends with that of energy savings using different species/strains of *Ceriporiopsis subvermispota* on loblolly pine chips (2-week incubation).

Species/strains	Blue	Yellowing of fiber ends		
		Slight	Inter	Advanced
Control	+			
<i>C. subvermispota</i> CZ-3	+ <sup>a</sup>	+ <sup>a</sup> (15%) <sup>b</sup>		
<i>C. subvermispota</i> L-14807 SS-1	+	+ (18%)		
<i>C. subvermispota</i> L-14807 SS-10		+	+ (22%)	
<i>C. subvermispota</i> FP-105752 SS-4			+ (22%)	
<i>C. subvermispota</i> L-14807 SS-5			+	+ (28%)
<i>C. subvermispota</i> L-14807 SS-3			+	+ (29%)

<sup>a</sup> Two pluses for one treatment indicate that the staining pattern was in between the two categories.

<sup>b</sup> Values in parentheses represent energy savings compared to the untreated control.

TABLE 4. Correlation of yellowing of fiber ends with that of energy savings using different levels of inoculum (% o.d. wt. basis) of *Ceriporiopsis subvermispota* strain CZ-3 on loblolly pine chips (2-week incubation).

Treatments	Blue	Yellowing of fiber ends		
		Slight	Inter	Advanced
Control	+			
0.01% inoculum	+ <sup>a</sup>	+ <sup>a</sup> (4%) <sup>b</sup>		
0.1% inoculum			+ (12%)	
0.3% inoculum				+ (19%)

<sup>a</sup> Two pluses for one treatment indicate that the staining pattern was in between the two categories.

<sup>b</sup> Values in parentheses represent energy savings compared to the untreated control.

surements have been reported previously (Akhtar et al. 1993).

#### *Simons stain*

This stain consists of 1% aqueous solution of Pontamine Sky Blue 6BX and 1% aqueous solution of Pontamine Fast Orange 6RN mixed in a 1:1 ratio. After the fibers on microscope slides are flooded with the stain, slides are heated at 60 C until evaporation. A cover glass is placed over the fibers, which are then rinsed with distilled water to remove excess stain. Fibers are then microscopically examined and immediately photographed. Pulp absorbs the orange stain if the fibers are fibrillated; fibers that are nonfibrillated stain blue. In this study, fibers obtained from biopulps under different experimental conditions showed different intensities of yellow stain, and were designated as slight, intermediate, or advanced. However, we would like to emphasize that this rating does not reflect any quantitative measurements and that the comparison should to be made only within each experimental set.

#### RESULTS

In order to establish a correlation between the Simons staining method and the energy savings during refining, pulp fibers were evaluated under different experimental conditions. For example, biopulp fibers were obtained from wood chips treated with different fungi, species, strains, and under different inoculum and nutrient conditions. All the control pulps stained a deep blue. The results obtained from these experiments are summarized below.

#### *Screening of different fungi*

In this experiment, loblolly pine chips were treated with *P. chrysosporium* and *C. subvermispota* for two weeks. Biopulp fibers from *P. chrysosporium* and *C. subvermispota* showed various yellow staining patterns, whereas controls were blue (Table 1). *P. chrysosporium* and *C. subvermispota* saved 9 and 16% energy, respectively, compared to the control (Table I).

#### *Screening of different species and strains of Ceriporiopsis*

Two different experiments were conducted: one with aspen chips (Table 2), and the other with loblolly pine chips (Table 3). In each case, incubation time was two weeks.

With aspen chips, biopulp fibers from *C. rivulosa* L-10602-sp-treated wood chips stained a deep blue just like the control fibers. However, biopulp fibers from *C. rivulosa* PiiRTO-26K225- and *C. pannocincta* FP-101181-sp-treated wood chips showed staining between blue and slight yellow; from *C. subvermispota* strains L-3292- and CZ-3-treated wood chips exhibited staining between slight and intermediate yellow; and from *C. subvermispota* strain L-9186-sp-treated wood chips had staining between intermediate to advanced yellow. Species showing blue coloration saved no energy, whereas species or strains showing coloration between blue and slight yellow saved 3–4% energy, between slight and intermediate yellow saved 7–12% energy, and between intermediate and advanced yellow saved 16% energy compared to the control (Table 2).

TABLE 5. Correlation of yellowing of fiber ends with that of energy savings using *Ceriporiopsis subvermispota* strain CZ-3 with and without medium on loblolly pine chips (4-week incubation).

Treatments	Blue	Yellowing of fiber ends		
		Slight	Inter	Advanced
Control	+			
<i>Ceriporiopsis subvermispota</i> CZ-3 (-medium)				+ (27%) <sup>a</sup>
<i>Ceriporiopsis subvermispota</i> CZ-3 (+medium)				+ (30%)

<sup>a</sup> Values in parentheses represent energy savings compared to the untreated control.

With loblolly pine, biopulps from strains CZ-3 and L-14807 SS-1 stained between blue and slight yellow, from strains L-14807 SS-10 and FP-105752 SS-4 stained between slight to intermediate yellow, and from strains L-14807 SS-5 and L-14807 SS-3 stained between intermediate to advanced yellow. Strains showing coloration between blue and slight yellow saved 15–18% energy, between slight to intermediate yellow saved 22% energy, and between intermediate to advanced yellow saved 28–29% energy compared to the control (Table 3).

#### Optimizing the inoculum levels and nutrient requirements

In these experiments CZ-3 strain of *C. subvermispota* was used on loblolly pine chips. Experiment with different inoculum levels was incubated for two weeks (Table 4), whereas experiment with and without the addition of medium was incubated for four weeks (Table 5).

Biopulp fibers obtained with the use of 0.01, 0.10, and 0.30% inoculum stained blue to slight yellow, intermediate yellow, and advanced yellow, respectively. Energy savings with these

inoculum levels were 4, 12, and 19%, respectively, compared to the control (Table 4). Biopulp fibers obtained in the presence or the absence of medium stained advanced yellow. Energy savings under these conditions were 27–30% compared to the control (Table 5).

#### Statistical correlation

Nonparametric Kendall's Tau Statistical test (Gibbons 1985) was used, which provides a measure of relationship or association between the variables that are measured on at least an ordinal scale, such as degree of yellowing experienced in the fiber ends. In this particular case, it is assumed that, within an experiment, seven levels of yellowing can be identified. For each experiment, the particular level of yellowing is then compared to the energy savings. A value of 1 for Kendall's Tau test indicates a positive association with perfect agreement between the degree of yellowing and energy savings, while a value of -1 indicates a negative association with perfect disagreement, or a value of zero indicates no detectable association. The *P*-value gives the probability of observing the absolute value of a particular Kendall's Tau Statistic assuming that there is no association between degree of yellowing and energy savings. This test showed a good association of fiber yellowing with energy savings for the experiments (Table 6). For the experiments summarized in Tables 1, 4, and 5 exact *P*-values can be calculated; for the experiments in Tables 2 and 3 the approximated *P*-values are given.

#### DISCUSSION

Our results clearly demonstrate that the intensity of yellow staining with Simons stain on biopulp fibers correlates very well with energy

TABLE 6. Nonparametric Kendall's Tau Statistic results showing relationship between the yellowing of fiber ends from biopulps and energy savings.

Experiments <sup>a</sup>	Kendall's tau statistic	Probability value ( <i>P</i> -value)
Table I	1.0000	0.3333 <sup>b</sup>
Table II	0.9747	0.0032
Table III	0.9234	0.0052
Table IV	1.0000	0.0833 <sup>b</sup>
Table V	0.8165	0.3333 <sup>b</sup>

<sup>a</sup> Correspond to table numbers describing experiment.

<sup>b</sup> Indicates exact value.

savings during biomechanical pulping. Our electron microscope studies suggested that biopulp fibers are highly fibrillated (Sachs et al. 1990). These results were confirmed in a recent publication (Blanchette et al. 1992a) where fibers from control and fungus-treated wood chips were observed microscopically after application of Simons stain. The edges of fibers from controls showed very little fibrillation, whereas those from biopulping experiments showed extensive fibrillation.

Refining of wood chips is a very time-consuming and labor-intensive process; therefore, staining of fibers after one pass through the refiner is an efficient method to speed up fungal screening and process optimization. In fact, we recently discovered that fungal pretreatments of wood chips on a very small scale (50 grams oven-dried chips), can be used to produce similar results to those from bioreactors that contained 1,500 grams oven-dried chips (Akhtar et al. unpublished). This protocol using a small amount of wood will further aid optimization processes.

The mechanism of action for staining patterns in fibers with Simons stain is not completely clear. Jayme and Harders-Steinhauser (1955) proposed that the blue dye has a smaller particle size than the yellow dye; and if fibers are not beaten, the small pore only allows the blue dye to penetrate. On the other hand, if fibers are beaten extensively and fibrillated, the pore size is larger, and the orange dye penetrates readily. Yu et al. (1995) showed that the response of fibers to the dyes is a measure of accessibility to the interior surface of the fibers and is independent of lignin concentration in various woods. We believe that fungal pretreatment modifies the woody cell wall in such a way that when the fibers are refined, the cut edges are frayed with fibrils that have been ripped from the wall. These fibrils absorb more of the high molecular weight orange dye. If such cell-wall modifications due to fungal action are intense, better penetration of orange dye in the biopulp fibers occurs and increased energy savings are realized. In this study, Simons stain has been used as a rapid visual screening tool. However, quantification of the

blue and orange dyes adsorbed in the fibers before and after the fungal pretreatment could also be studied so that the percent energy savings after various treatments and optimization procedures can be made with even greater accuracy.

#### CONCLUSIONS

Simons stain can serve as a rapid screening tool for evaluating the biopulping efficacy of different species and strains of white-rot fungi, and it can also be used to determine optimum conditions for the selected fungi. This rapid screening method will help in identifying the best strains and procedures for use in commercial biopulping processes.

#### ACKNOWLEDGMENTS

This research was supported in part by the Biopulping Consortia I and II, which consisted of pulp and paper and associated companies. It is paper number 21467 of the Minnesota Agricultural Experiment Station, St. Paul, Minnesota 55108.

#### REFERENCES

- AKHTAR, M. 1994. Biomechanical pulping of aspen wood chips with three strains of *Ceriporiopsis subvermispora*. *Holzforschung* 48:199–202.
- , M. C. ATTRIDGE, R. A. BLANCHETTE, G. C. MYERS, M. B. WALL, M. S. SYKES, J. W. KONING, JR., R. R. BURGESS, T. H. WEGNER, AND T. K. KIRK. 1992a. The white-rot fungus *Ceriporiopsis subvermispora* saves electrical energy and improves strength properties during biomechanical pulping of wood. Pages 3–8 in M. Kuwahara and M. Shimada, eds. *Biotechnology in pulp and paper industry*. Kyoto, Japan.
- , ———, G. C. MYERS, T. K. KIRK, AND R. A. BLANCHETTE. 1992b. Biomechanical pulping of loblolly pine with different strains of the white-rot fungus *Ceriporiopsis subvermispora*. *Tappi* 75:105–109.
- , ———, ———, AND R. A. BLANCHETTE. 1993. Biomechanical pulping of loblolly pine chips with selected white-rot fungi. *Holzforschung* 47:36–40.
- , G. F. LEATHAM, G. C. MYERS, AND M. C. ATTRIDGE. 1989. PFI milling: A possible method to predict both energy savings and paper strength properties in biomechanical pulping. Paper presented at the Fourth International Conference on Biotechnology in the Pulp and Paper Industry. May 16–19, Raleigh, NC.
- BLANCHETTE, R. A., M. AKHTAR, AND M. C. ATTRIDGE. 1992a. Using Simons stain to evaluate fiber characteristics of biomechanical pulps. *Tappi* 75:121–124.

- , T. A. BURNES, M. E. EERDMANS, AND M. AKHTAR. 1992b. Evaluating isolates of *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispota* for use in biological pulping processes. *Holzforschung* 46:109–115.
- FISCHER, K., M. AKHTAR, R. A. BLANCHETTE, T. A. BURNES, K. MESSNER, AND T. K. KIRK. 1994. Reduction of resin content in wood chips during experimental biological pulping processes. *Holzforschung* 48:285–290.
- GIBBONS, J. D. 1985. Nonparametric methods for quantitative analysis. American Sciences Press, Columbus, OH.
- JAYME, G., AND M. HARDERS-STEINHAUSER. 1955. Über die erkennung von dichteunterschieden in cellulosefasern durch doppelfärbung. *Das Papier* 9(21/22):507–510.
- KIRK, T. K., J. W. KONING JR., R. R. BURGESS, M. AKHTAR, R. A. BLANCHETTE, D. C. CAMERON, D. CULLEN, P. J. KERSTEN, E. N. LIGHTFOOT, G. C. MYERS, M. SYKES, AND M. B. WALL. 1993. Biopulping: A glimpse of the future? Res. Rep. FPL-RP-523, Madison, WI.
- LEATHAM, G. F. 1983. A chemically defined medium for the fruiting of *Lentinus edodes*. *Mycologia* 75:905–908.
- , AND G. C. MYERS. 1990. A PFI mill can be used to predict biomechanical pulp strength properties. *Tappi* 73(4):192–197.
- , ———, T. H. WEGNER, AND R. A. BLANCHETTE. 1990a. Biomechanical pulping of aspen chips: Paper strength and optical properties resulting from different fungal treatments. *Tappi* 73(3):249–255.
- , ———, ———, AND ———. 1990b. Energy savings in biomechanical pulping. Pages 17–25 in T. K. Kirk and H.-M. Chang, eds. *Biotechnology in pulp and paper manufactures, applications and fundamental investigations*. Butterwoths-Heinemann, Boston, MA.
- SACHS, I. B., G. F. LEATHAM, G. C. MYERS, AND T. H. WEGNER. 1990. Distinguishing characteristics of biomechanical pulp. *Tappi* 73(9):249–254.
- SIMONS, F. L. 1950. A stain for use in the microscopy of beaten fibers. 1950. *Tappi* 33(7):312–314.
- SYKES, M. 1994. Environmental compatibility of effluents of aspen biomechanical pulps. *Tappi* 77(1):160–166.
- WURZ, O. 1969. Studying the degree of beating of chemical pulps with the Simons stain. *Paper Maker* 38:59.
- YU, X., J. L. MINOR, AND R. H. ATALLA. 1995. Mechanism of action of Simons stain. *Tappi* (in press).