Fungal Delignification and Biomechanical Pulping of wood

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This review article summarizes the results on microstructural changes and delignification, lignin-degrading enzyme systems, and biopulping of wood with lignin-degrading fungi. Biopulping, defined as the treatment of wood chips with lignin-degrading fungi prior to pulping, saves substantial amount of electrical energy during mechanical pulping, results in stronger paper, and lowers the environmental impact of pulping. Optical properties are diminished; however, brightness can be restored readily with peroxide bleaching. The economics of the process look attractive if the process can be performed in a chip-pile based system. Past work on biopulping had been minimal, however a comprehensive evaluation of biopulping at the Forest Products Laboratory suggests that biopulping has a good chance of commercial success.

Advances in Biochemical Engineering/
Biotechnology, Vol. 57
Managing Editor: T. Scheper

1 Fungal Degradation of Wood

1.1 Introduction

Although wood can be attacked by an array of microorganisms, fungi are the predominant decomposes in terrestrial ecosystems. Extracellular enzymes produced by decay fungi and subsequent decomposition processes differ among the various groups of fungi, resulting in different types of wood degradation. Brown, soft, and white rot are categories used to separate different forms of decay. These categories are based on the macroscopic characteristics of the advanced stages of decay. Brown rot is caused by fungi taxonomically classified in the Basidiomycotina. These fungi cause rapid and extensive depolymerization of cellulose early in the decay process [1-4]. Wood polysaccharides are degraded, lignin modification occurs, and relatively small amounts of lignin are lost as decay progresses [5–7]. In advanced stages of decay, the residue is a brown mass, which mostly consists of lignin. This decayed wood is sponge-like when wet, but often cracks and checks into cubical pieces as the wood dries. Brown-rot fungi are common decomposes in conifer forests and also are responsible for most decay found in buildings and wood in service.

Soft rot is a term first used to describe decay by fungi attacking wood surfaces in wet environments [8]. Fungi in the Ascomycotina and Deuteromycotina attack wood surfaces resulting in soft, gray to brown decay. Since soft rot was first reported, knowledge of these organisms has expanded—soft rot fungi have been found associated with wood in many different situations, such as in wood treated with preservatives or in wood that receives only intermittent moisture [6, 9–11]. Two forms of soft rot attack, Type I and Type II, have been identified [12]. Type I consists of cavities within the secondary wall. Mycelia in cell lumina produce fine penetrating hyphae that enter the secondary wall, align growth along the microfibrillar axis of the cell wall, and produce chains of cavities. The Type II form of attack consists of an erosion of the entire secondary wall originating from hyphae in cell lumina and progressing toward the middle lamellae. The entire secondary wall maybe degraded, but the middle lamella is not attacked. Certain fungi, including *Hypoxylon* spp. and *Xylaria* spp., were previously considered to be white-rot fungi, but their mode of attack is more correctly classified a Type II form of soft rot [6, 13].

Fungi that cause white rot belong to the Basidiomycotina and have the capacity to degrade all cell wall components, including lignin. The extent of lignin degradation can vary considerably among species of white-rot fungi [14]. Some species, such as *Trametes versicolor,* are nonselective in how they degrade the wood, i.e., they simultaneously degrade lignin, cellulose, and hemicelluloses. Other species, such as *Phellinus pini, Ceriporiopsis subvermispora,* and *Phlebia tremellosa,* cause preferential degradation of lignin [15]. Some species deplete lignin, cellulose, and hemicellulose in varying ratios; many species attack both nonselectively and selectively in different areas of the same substrate [14].

Although white-rot fungi have been categorized by whether they cause selective lignin degradation or nonselective decay, it is apparent that the degradation processes of these fungi are extremely variable. Even different strains of one species of white-rot fungus were recently shown to degrade cell wall components differentially [16].

1.2 Wood Decay by White-Rot Fungi

1.2.1 Microstructural Changes and Delignification

White-rot fungi enter cell lumina and rapidly colonize ray parenchyma cells that contain free sugars and other nutrients. The radial arrangement of the ray parenchyma facilitates access into the wood and allows widespread distribution of the fungus in the substrate. Access to adjacent cells occurs via pit apertures, or direct penetration may take place directly through the cell wall [2, 4]. Once easily-assimilated substances are depleted, degradation of the cell wall is initiated. White-rot fungi that attack all cell wall components simultaneously cause a localized erosion of all cell wall layers. The attack progresses through the secondary wall layers and middle lamella (Fig. 1A and B). In advanced stages of decay, cell walls are eroded extensively, and holes within adjacent cell walls are frequently observed (Fig. 1B). A different form of cell wall attack occurs in white-rot fungi that selectively degrade lignin. Hyphae in cell lumina degrade lignin progressively from the lumen edge of the secondary wall toward the middle lamella (Fig. 1C and D). Investigations using brominated wood and X-ray microanalysis of the bromine–lignin complex showed that white-rot fungi remove lignin from the secondary wall before the middle lamellae between cells are degraded [17,18]. As the delignification process continues, the middle lamella is degraded and cells separate from adjacent cells (Fig. 1C and D). The delignified, cellulose-rich secondary wall remains relatively unaltered (Fig. 1C and D). The degradation of lignin is extensive throughout the cell walls, originating from only one or two hyphal filaments within each cell lumen (Fig. 2A and B).

Wood degradation may be influenced significantly by the lignocellulosic substrate. An important factor that governs the extent and rate of decay is the amount and type of lignin present in the wood. Wood from gymnosperms has greater concentrations of lignin than wood from angiosperms and consists primarily of guaiacylpropyl units. Lignin from angiosperm wood is composed of varying amounts of syringylpropyl and guaiacylpropyl units. In studies evaluating decay by white-rot fungi in different types of wood, angiosperm wood was found to degrade more rapidly and to a greater extent than gymnosperm wood [1, 15, 19]. Synthetic syringyl lignin has also been shown to be depolymerized more rapidly than synthetic guaiacyl lignin in a laboratory investigation using *Phanerochaete chysosporium* [20].

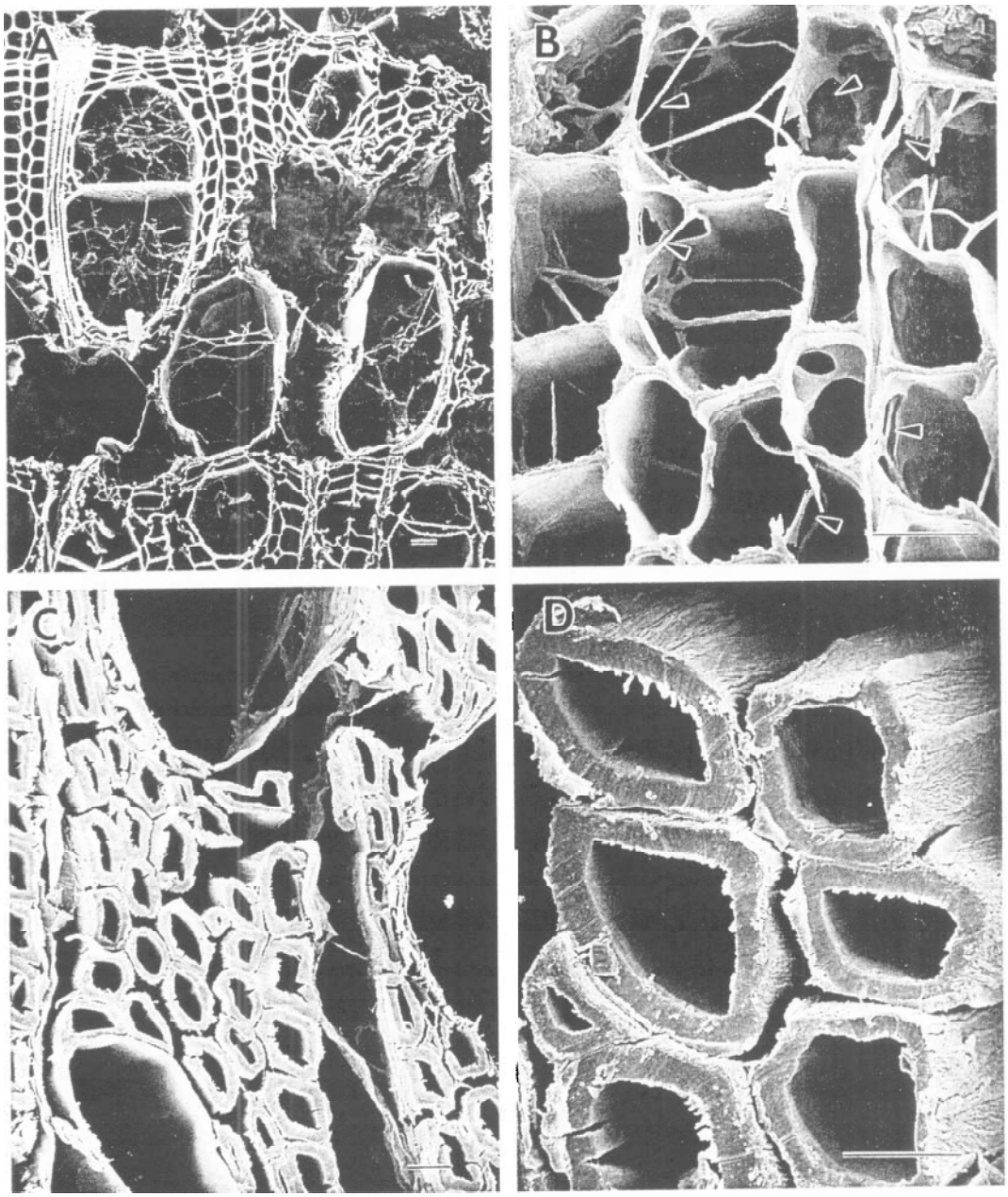

Fig. 1. Wood decayed by white-rot fungi with simultaneous removal of all cell wall components **(A** and **B)** and preferential degradation of lignin **(C** and **D).** A nonselective attack erodes all cell wall layers, including the compound middle lamellae. Erosion zones coalesce to form holes through cell walls (arrows). **C** and **D:** Lignin in secondary walls and middle lamella regions has been removed by selective delignification. Cells separate from adjacent cells as a result of the lack of middle lamellae. The cellulose-rich secondary wall remains. Transverse sections, scanning electron micrographs. *Bar* = 10mm

Delignification in angiosperms by white-rot fungi also appears to be influenced by the syringyl lignin content of the wood. Studies of wood decay in *Nothofagus dombeyi* from the temperate rain forests of southern Chile showed extensive delignification by *Ganoderma australe* [14, 21]. This is one of the few locations in the world where huge logs may be completely delignified. In

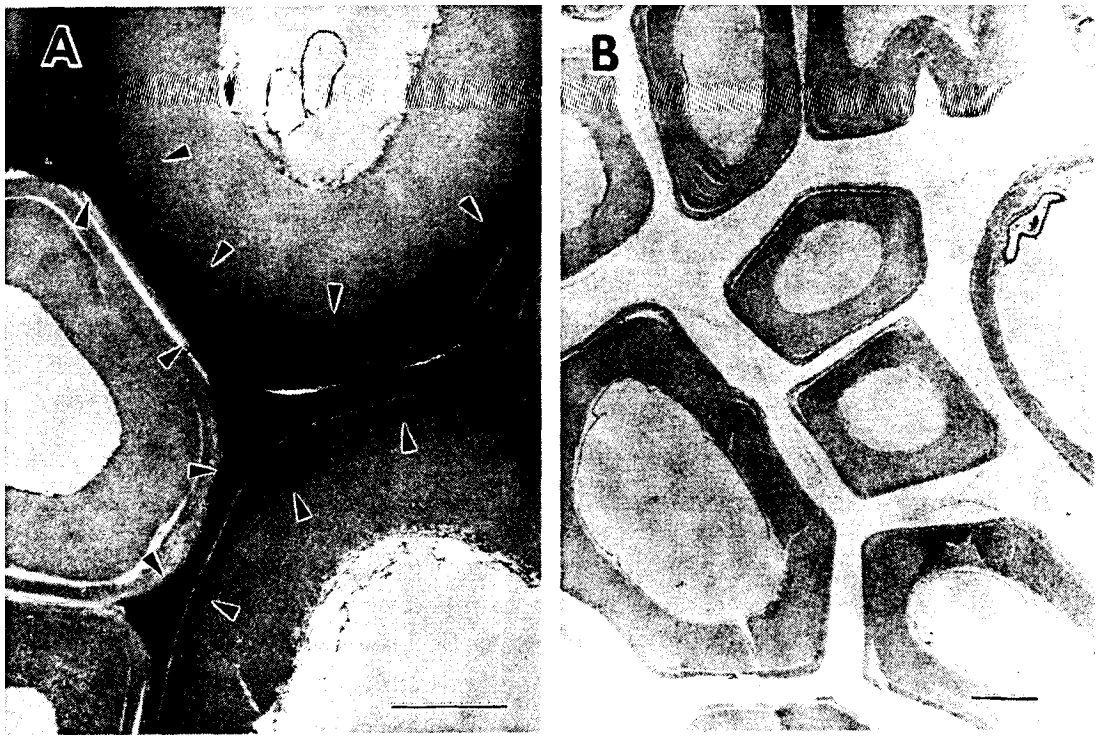

Fig. 2A, B. Selective delignification in cell walls appears as electron-lucid zones after $KMnO_4$ fixation and transmission electron microscopy. **A** Lignin is progressively removed from the secondary wall and then from the middle lamella (arrows show extent of delignification). **B:** Completely delignified cells-detached cells free of middle lamellae. *Bar* = 5 mm

temperate forests of North America, Europe, and Japan, delignification usually occurs in small pockets within the wood or sometimes in large localized zones, but the delignification of entire logs has not been reported. *Nothofagus dombeyi* has an extremely high syringyl lignin content [21] —three to six times that of *Acer, Betula,* and other hardwood species—and, worldwide, it appears to be among woods with the highest content of syringyl lignin. Other angiosperms growing in southern Chile, such as *Laurelia phillipiana,* that have low syringyl-to-guaiacyl lignin ratios are not delignified when decayed by *G. australe.* Instead, a nonselective white-rot type of degradation occurs [21]. These results strongly suggest that the concentration of syringyl lignin within angiosperm wood greatly influences the delignification process.

Although white-rot fungi preferentially degrade syringyl lignin in angiosperms, some white-rot species extensively degrade guaiacyl lignin in gymnosperm. Fungi such as *P. pini, Heterobasidion annosum,* and *P. weirii* are usually found only in coniferous wood and apparently have evolved highly efficient mechanisms of lignin degradation. Other species of white-rot fungi also do not appear to be deterred by the guaiacyl lignin of pines and other conifers. Species such as *C. subvermispora* can readily delignify the sapwood of loblolly pine, causing considerable amounts of lignin removal [16]. These species are of special interest for use in biological pulping processes because they can degrade lignin in conifers as well as hardwoods.

The process of preferential delignification of woody cell walls by white-rot fungi has been elucidated using a variety of techniques. Histological stains and electron-dense compounds that react with lignin can be used to visualize lignin removal from the cell wall [22, 23]. $KMnO_4$ can be used to fix wood for transmission electron microscopy and to observe the distribution of lignin in cell walls. Moderate electron density is evident in the secondary wall where lignin is found with cellulose and hemicellulose, but intense electron density can be observed in the highly lignified middle lamellae [17]. As hyphae of white-rot fungi, located in cell lumina, begin to delignify the secondary wall, an electron-transparent zone develops (Figs. 2A). This clear zone progressively moves into the secondary wall. Once lignin is removed from this region, the middle lamella between cells and, in very advanced stages of decay, the cell corner regions are degraded and become less electron dense (Fig. 2B) [14].

A light-based microscopic method that employs bright colored stains (astra-blue and safranin) has recently been used to differentiate zones of delignification from nondecayed cells that retain lignin [24]. Colloidal gold cytochemistry using gold-labeled *endo*- 1,4-b-glucanase II, 1-4-b-D-glucan cellobiohydrolase I, and endo- 1,-4-b-xylanase revealed that residual delignified wood contains crystalline and amorphous cellulose but little xylan [25, 26]. Other studies also have shown that hemicelluloses are usually depleted from wood as lignin is being degraded [14, 27].

1.2.2 Lignin-Degrading Enzyme Systems

Lignin-degrading enzymes were discovered, and subsequently characterized, in *P. chrysosporium* (28–32). Research on the biochemistry of lignin degradation began in earnest in the 1970s, when ^{14}C-lignins were prepared and used to determine which groups of microbes are able to mineralize lignin (decompose it to $^{14}CO_2$). The higher basidiomycetous fungi were found to be the most proficient, and *P. chrysosporium* was chosen for detailed study for a number of reasons related to ease of experimentation, Subsequent research with this fungus described the culture parameters important for lignin mineralization and showed that one or more steps are “secondary metabolic” events triggered by limitation for certain nutrients. At the same time, the chemical changes that occur in the lignin polymer during decay by ligninolytic fungi were described (reviewed by Kirk and Farrell [29]). In the early 1980s, specific degradative reactions accomplished by ligninolytic cultures of *P. chrysosporium* were described using synthesized “dimeric” lignin model compounds; it was also found that certain polymeric dyes are decolonized. Cell-free enzyme preparations that catalyzed dimer cleavage and dye decolonization were reported in 1983, and subsequently the responsible peroxidase (lignin peroxidase) was described.

Evidence to date indicates that three oxidizing enzymes, lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase are responsible for the initial fragmentation of the lignin polymer and production of low molecular mass

breakdown products in white-rot fungi. Not all white-rot fungi apparently produce all three enzymes, although some, including *T. versicolor,* do. *P. chrysosporium* produces only LiP and MnP (however, laccase has recently been reported in *P. chrysosporium* [33]), whereas *C. subvermispora* produces only MnP and laccase, and *Phlebia ochraceofulva* produces only LiP and laccase [32]. The extracellular H_2O_2 required as electron acceptor for the peroxidases is supplied by glyoxal oxidase, which, with its substrates, is extracellular, and perhaps also by intracellular sugar and alcohol oxidases. Molecular oxygen is the electron donor for laccase. Low molecular mass products of lignin degradation are taken up by the hyphae and further oxidized. The enzymes responsible for this intracellular oxidation are only beginning to be described [34].

Lignin peroxidase oxidizes aromatic nuclei (phenolic and nonphenolic) by removal of one electron, generating both phenoxy radicals and cation-radicals. The latter react spontaneously with nucleophiles (primarily water) and molecular oxygen. The result is an "enzymatic combustion" in which C-C and C-O linkages are cleaved, depolymerizing the polymer and opening aromatic rings. A plethora of aromatic and aliphatic products are thereby formed. In vitro depolymerization of lignin by pure LiP in the presence of H_2O_2 and veratryl alcohol was recently demonstrated [35]. Veratryl alcohol (3,4-dimethoxybenzyl alcohol), found in the extracellular milieu of ligninolytic cultures of examined LiP-producing fungi, is a secondary metabolic product synthesized *de novo.* It is a substrate for LiP and stimulates its action, probably not as an electron mediator as originally thought (see Kirk and Farrell [29]), but by donating electrons to LiP so that its catalytic cycle is completed [36]. As mentioned, LiP is apparently not produced by some white-rot fungi, including *C. subvermispora* [37], suggesting that it is not required in all fungi, i.e., that the white-rot fungi have more than one enzyme system for degrading lignin. Interestingly, *C. subvermispora* and certain other LiP-negative fungi do have "LiP-like" genes [37].

Manganese peroxidase has been found in nearly all studied white-rot fungi. It catalyzes the oxidation of (complexed) Mn^{2+} to Mn^{3+}, which in turn oxidizes lignin. Mn^{2+} is a fairly abundant element in wood. The "physiological" complexes that have been studied, such as the lactate chelate, oxidize only phenolic units, which constitute only about 10% of the total in lignin in wood [38]. The phenolic units are oxidized to phenoxy radicals, which can undergo certain degradative reactions [39]. The $MnP/H_2O_2/Mn2^+$ has been shown to depolymerize lignin in vitro [40]. The exact function of MnP and the chemistry of its actual oxidation of lignin are not yet clear.

In a recent study, Bao et al. [41] discovered a lipid peroxidation system involving MnP. In the presence of Mn(II), MnP promotes the peroxidation of unsaturated lipids, generating transient lipoyl radical intermediates that are known to act as potent oxidants of other molecules. This system, unlike MnP alone, oxidizes and cleaves nonphenolic model compounds via benzylic hydrogen abstraction. It also depolymerizes both nonphenolic and phenolic synthetic lignins, which strongly supports a ligninolytic role for this system in vivo. The

ligninolytic system of *P. chrysosporium* is depicted schematically in Fig. 3. We have included the lipid peroxidation system, even though its role is not yet established.

Laccaseis a blue copper oxidase that is produced (secreted) by most but not all white-rot fungi. Like LiP, laccase is apparently not required for lignin degradation in all fungi. Laccase oxidizes phenolic units in lignin to phenoxy radicals, which is the same process as that brought about by the chelated Mn(III) produced by MnP (Fig. 3). However, in the presence of appropriate "primary'' substrates (such as ABTS), the effect of laccase apparently can be enhanced; laccase/primary substrate systems have recently been reported to degrade ligninin kraft pulp [42] and to oxidize nonphenolic compounds that otherwise are unattached by laccase. However, it is not known whether such "primary'' substrates occur and function in vivo, and the actual role of laccase, like MnP, remains unclear. (Note that if "primary" substrates augment laccase activity in vivo, they can be expected to augment the MnP/Mn^{2+} system as well).

The characteristics of LiP, MnP, glyoxal oxidase, and laccase have been described in review articles [28–31, 39, 43]. General properties of LiPs, common to all studied LiP-producing fungi, include the following. Lignin peroxidases consist of acidic isoenzymes encoded by multiple structural genes whose expression

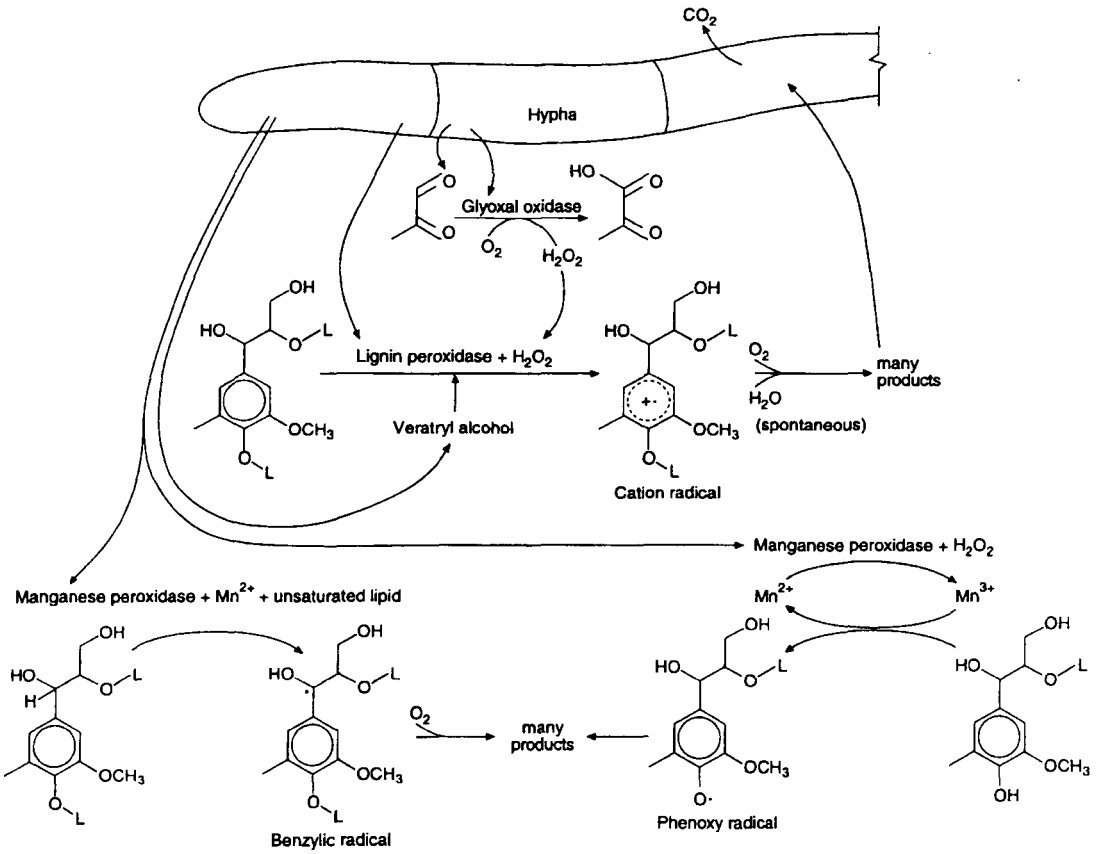

Fig. 3. Scheme depicting the lignin-degrading system of *Phanerochaete chrysosporium*

is nutrient-regulated. The extracellular proteins have protoporphyrin IX-type heme prosthetic groups, are glycosylated, and have molecular weights around 40 kDa. Lignin peroxidases exhibit the common peroxidase catalytic cycle, being first oxidized by H_2O_2 by removal of two electrons to give compound I, which oxidizes its substrates by removing one electron to give compound II, which oxidizes its substrate, returning to resting enzyme. The LiP from *P. chrysosporium* has been crystallized and its three-dimensional structure resolved by X-ray crystallography [44, 45].

Manganese peroxidases are like LiP in that they consist of multiple acidic isoenzymes encoded by multiple structural genes whose expression is nutrient-regulated; in the case of MnP, regulation by Mn^{2+} also occurs. Manganese peroxidases are slightly larger than LiPs, but exhibit the same basic peroxidase catalytic cycle. Interestingly, MnP, in the presence of reducing agents such as glutathione, transfers electrons to molecular oxygen, generating H_2O_2. Like LiP, MnP has been crystallized and its three-dimensional structure determined [46]. Note that LiP can display MnP activity; in the presence of H_2O_2, O_2, and the metabolizes veratryl alcohol and oxalate, LiP oxidizes Mn^{2+} to Mn^{3+} [38].

In at least some white-rot fungi, including *T. versicolor,* laccases also consist of acidic isoenzymes encoded by multiple structural genes. The molecular weights of the laccases of white-rot fungi are in the range 50-65 kDa. Laccases "store" four electrons from four sequential one-electron oxidations before reducing molecular oxygen to water.

The molecular genetics of the lignin-biodegrading system have received attention in the last few years. Such studies have now been done with several fungi, although *P. chrysosporium* has been the most thoroughly studied. With that organism (and with the other studied fungi as well), complex structural gene families have been found. In *P. chrysosporium,* 10 *lip,* 4 *mnp,* and 1 *glox* have been cloned and sequenced. All the genes contain introns and multiple glycosylation sites. Further detail is found in recent reviews [31, 43].

1.2.3 Enzyme Localization

Immunological cytochemistry in conjunction with electron microscopy can be used to localize enzymes within substrates and observe their spatial relationship within decaying wood. The association of delignified zones with specific enzymes would indicate that the enzymes were able to penetrate the cell wall and accumulate at sites of lignin degradation. Investigations using polyclonal and monoclinal antibodies to lignin peroxidase and manganese peroxidase have shown that these enzymes are present in cell walls of wood decayed for 6 to 12 weeks [25, 47-49]. High concentrations of enzymes were found in decayed cell walls that had a loose, modified ultrastructural matrix. Lignin peroxidase and manganese peroxidase were evident at the edge of electron-dense regions in the secondary wall or at sites of middle lamella degradation. Significant alteration of the cell wall was always associated with localization of these enzymes. No

labeling was evident in nondecayed wood or in wood decayed by a brown-rot fungus [25].

To more accurately identify areas of the cell wall where enzymes could penetrate successfully, several ultrastructural studies were completed after infiltrating decayed wood with extracellular extracts from white-rot fungi or with purified lignin peroxidase and manganese peroxidase [48, 50]. After impregnation, gold-labeled antibodies were used to determine the extent of penetrability in the cells. In nondegraded cells, no enzyme preparations were found to enter the secondary wall. Enzymes were found only on the surface of the cell wall at the lumen. In samples decayed by white-rot fungi, lignin peroxidase and manganese peroxidase were found within altered cell walls. The enzymes diffused into the peripheral areas of the secondary wall and into areas where the middle lamella was becoming less electron-dense. The labeling appeared to be located at sites where lignin was being degraded. In areas with advanced decay, enzymes were located within the cell wall at the edges of undegraded cell corner regions [48].

2 Biopulping

2.1 Introduction

The pulp and paper industry is a large and growing portion of the world's economy. In 1991, paper sales were valued at $122 billion [51], and 267 million metric tons of paper and paperboard were consumed worldwide. Worldwide consumption is expected to increase to 300 million metric tons in 1996. A number of pulping processes have been developed to meet industrial and consumer needs.

Pulping processes are generally divided into two broad classes, chemical and mechanical, which produce substantially different fiber characteristics. The choice of process depends on the end application of the pulp and the raw material. In many papermaking operations, a combination of chemical and mechanical pulps is used to obtain the desired paper characteristics.

Chemical pulping involves the use of chemicals to degrade and dissolve the lignin from the wood cell walls, releasing cellulose fibers. Chemical pulping processes are low yield (about 40-50%) and require significant waste treatment and chemical recycling operations; however, the pulps produced have high strength. Mechanical pulping involves the use of mechanical force to separate the wood fibers. Mechanical processes are high yield (up to 95%) and give paper with high bulk, good opacity, and excellent printability. However, these processes are energy-intensive and produce paper with lower strength and high color reversion (tendency to turn yellow with time).

Bleaching of chemical pulps using a combination of chlorination and alkaline extraction has been used in the pulp and paper industry for many years.

Unfortunately, the effluents from chlorination and alkaline extraction stages cannot be recycled back to the chemical recovery furnace because of their high level of corrosive chloride. In addition, the effluents contain large amounts of chlorinated organic compounds, which are known to have toxic, mutagenic, and carcinogenic effects. In most parts of the world, increased public concern about the environment is having a large impact on the pulp and paper industry. Reductions in allowable air and water discharges from pulp and paper mills are requiring restraint in the use of chlorine and chlorine dioxide for bleaching. Consequently, the industry is rapidly moving towards alternative technologies to alleviate this and other problems related to the environment.

Biopulping, defined as the treatment of wood chips with lignin-degrading fungi prior to pulping, is an experimental process that has been researched extensively during the past 8 years. It has been studied mainly as a pretreatment for mechanical pulping. Biopulping reduces electrical energy consumption (which is the major cost in mechanical pulping), improves paper quality, and reduces the environmental impact of pulping [52, 53]. The following sections present a summary of this research and describe key findings.

2.2 Past Work

The use of white-rot fungi for the biological delignification of wood was perhaps first studied by Lawson and Still [54] at the West Virginia Pulp and Paper Company research laboratory (non Westvaco Corporation). These researchers published a survey of the literature (covering 72 lignin-degrading fungi), which pointed to the dearth of knowledge about the fungal degradation of lignin. Research was then done at the US Forest Products Laboratory in Madison and the Swedish Products Laboratory (STFI) in Stockholm. The first published report on biopulping per se demonstrated that fungal treatment could result in significant energy savings for mechanical pulping [55]. That research resulted in a US patent [56], which described a “method for producing cellulose pulp.”

Considerable efforts at STFI were directed toward developing cellulase-less mutants of selected white-rot fungi to improve the selectivity of lignin degradation and thus the specificity of biopulping [57]. However, the mutant strains degraded less lignin than did wild-type strains when grown on wood [58] and did not result in energy savings during subsequent mechanical pulping [59]. Attempts by this group to scale up the biopulping process were not notably successful [60]. However, subsequent work with Cuban scientists on a pilot scale with bagasse using mutant strains gave more promising results [61]. Eriksson et al. [62] showed that chip colonization is not the rate-limiting step in biopulping. At the Forest Products Laboratory, Bar-Lev et al. [63] showed that the treatment of primary thermomechanical pulp with a white-rot fungus prior to secondary refining reduced energy requirements and increased paper strength properties. Similar results were obtained in Japan by Akamatsu et al. [64] during thermomechanical pulping of fungus-treated popular chips. Other

details on biopulping research were described in two review articles and the literature cited therein [65, 66].

A comprehensive evaluation of biomechanical pulping was launched in 1987 at the Forest Products Laboratory after the establishment of a Biopulping Consortium, which involved the Forest Products Laboratory, the Universities of Wisconsin and Minnesota, and pulp and paper and related companies. The overall goal was to establish the technical feasibility of using a fungal pretreatment with mechanical pulping to save energy and/or improve paper strength. In addition, it was assumed that the fungal pretreatment would have less environmental impact than would chemical pretreatments, which turned out to be the case. The consortium research was conducted by seven closely coordinated teams: fungal, pulp and paper, enzyme, molecular genetics, economics, engineering scale-up, and information. However, in this review article, we will focus only on the work conducted by the fungal pulp and paper, and engineering scale-up research teams.

2.3 Biomechanical Pulping

2.3.1 Screening of Fungi

There are hundreds of white-rot fungi with varying capacities to degrade lignin, cellulose, and hemicellulose. We assumed at the outset that the fungi that degraded lignin selectively would be the best candidates for biopulping. To ascertain the most appropriate species, a screening program was initiated that selected fast-growing species that could selectively remove lignin from wood.

2.3.1.1 In Vitro Wood Decay Test

Several methods have been developed to select fungal species with selective lignin-degrading ability [15, 27, 67–70]. However, one of the most appropriate methods appeared to be an assessment of decay (chemical analyses of lignin and wood sugar content) using wood blocks in accelerated decay chambers [15, 71]. Based on this in vitro screening procedure, we selected several species of fungi; among the best were *P. chrysosporium, C. subvermispora, Phlebia brevispora, Phlebia tremellosa, Dichomitus squalens,* and *Phellinus pini* [15, 71]. Different strains of these selected species varied in their selectivity towards lignin. Two fungi were examined in detail: *P. chrysosporium* and *C. subvermispora.* As we tested various strains on different species of wood, it became clear that some strains are effective with hardwood only, whereas others are effective on both hardwood and softwood (Tables 1-4). These results clearly showed large differences among the strains in capacity to degrade lignin and in selectivity [16].

The species or strains selected by this method were then evaluated for their biopulping efficacy. No apparent relationship was found between the lignin removal from the wood chips and energy savings or strength improvements during the actual biopulping runs [72, 73]. This suggests that lignin

Table 1. Loss of weight, lignin, and wood sugars in aspen wood blocks decayed by different strains of *P. chrysosporium* (12-week incubation)

	Loss (%.)				
Strain	Weight	Lignin	Glucose (glucan)	Xylose (xylan)	Mannose (mannan)
BKM-F-1767	61.0	80.7	49.9	77.7	61.0
5161ME-8	53.6	54.4	53.0	61.0	77.6
FP-104297-sp	55.3	47.5	51.3	63.0	81.5
FP-102169	50.2	56.7	51.4	52.0	55.3
HHB-6251-sp	56.2	58.9	55.0	59.4	59.2
5157-A-1	23.0	30.6	5.2	27.4	60.2
Gold-9-420-1	61.2	73.4	56.1	71.7	67.9

Table 2. Loss of weight, lignin, and wood sugars in loblolly pine wood blocks decayed by different strains of *P. chrysosporium* (12-week incubation)

	Loss (%)				
Strain	Weight	Lignin	Glucose (glucan)	Xylose (xylan)	Mannose (mannan)
BKM-F-1767	24.5	20.9	26.1	19.1	31.4
5161ME-8	8.4	3.6	11.2	9.7	14.5
FP-104297-sp	22.7	12.9	30.5	23.8	39.3
FP-102169	19.7	16.9	23.0	17.4	24.3
HHB-6251-sp	11.9	8.3	8.7	6.9	5.3
5157-A-1	13.6	12.8	10.3	14.8	11.5
Gold-9-420-1	18.7	18.3	17.5	25.7	20.7

Table 3. Loss of weight, lignin, and wood sugars in aspen wood blocks decayed by different strains of *C. subvermispora* (l2-week incubation)

	Loss (%)				
Strain	Weight	Lignin	Glucose (glucan)	Xylose (xylan)	Mannose (mannan)
ME-485	28.4	61.5	2.5	45.4	72.3
L-14807-sp	24.4	57.2	6.8	36.9	39.1
L-15225-sp	25.4	58.8	2.9	40.4	66.3
FP-104027-T	26.4	65.9	2.2	44.6	66.8
L-39292-sp	25.6	63.7	2.1	47.9	66.4
FP-105752-sp	22.7	55.7	0.6	31.0	30.2
CZ-3	23.8	71.2	6.3	43.8	28.7
L-6133-sp	24.4	70.7	3.4	38.4	29.3
FP-90331-sp	26.5	50.1	7.3	31.5	31.3

Table 4. Loss of weight, lignin, and wood sugars in loblolly pine wood blocks decayed by different strains of *C. subvermispora* (12-week incubation)

	Loss (%)				
Strain	Weight	Lignin	Glucose (glucan)	Xylose (xylan)	Mannose (mannan)
ME-485	22.8	31.0	20.3	0	24.2
L-14807-sp	22.5	37.0	14.7	33.2	29.9
L-15225-sp	23.7	38.2	12.4	27.2	28.1
FP-104027-T	28.3	40.6	18.8	33.8	26.9
L-39292-sp	29.0	42.2	22.4	31.1	26.2
FP-105752-sp	19.6	33.9	7.1	27.0	10.1
CZ-3	21.3	31.8	14.0	31.0	20.3
L-6133-sp	30.1	34.1	26.2	34.0	18.9
FP-90331-sp	22.7	38.2	14.1	30.0	15.9

modification rather than removal is involved. Screening by way of the in vitro wood decay test is also time-consuming and labor-intensive. We therefore sought alternative screening methods.

2.3.1.2 PFI Milling

PFI milling and freeness measurements have previously been used to assess energy consumption of fungus-treated pulps [74]. We further evaluated this approach and tried to correlate changes in freeness after PFI mill refining of coarse aspen pulp treated with selected white-rot fungi with those of paper strength properties or energy savings obtained during biomechanical pulping of wood chips with the same fungi. We found that PFI milling and freeness measurements of pulp can, in themselves, give a good estimate of paper strength properties [75]. However, follow-up studies suggested that this method can only be used to evaluate the effect of fungal treatments on energy savings compared to the control; they cannot be used to discriminate the effect of different fungal treatments on energy savings [76].

2.3.1.3 Simons Staining

Simons stain [77, 78] had been used previously in various investigations to evaluate the degree of fibrillation in beaten pulp fibers. The stain consists of a 1% aqueous solution of Pontamine Sky Blue 6BX and a 1% aqueous solution of Potamine Fast Orange 6RN mixed in a 1:1 ratio. Fibers on microscope slides are first flooded with stain and then heated at 60°C to evaporate the water. The fibers are rinsed with distilled water to remove excess stain and then covered with glass. The fibers are then immediately examined under a microscope and photographed. Fibrillated fiber walls stain orange, and nonfibrillated or undamaged fiber walls stain blue.

Our earlier electron microscope studies in connection with biopulping had shown that pulp fibers obtained from the fungus-treated wood chips had more uniform fiber length and greater fibrillation, and appeared woolly compared

with control pulps [79], suggesting that extent of fibrillation might be a good indicator of biopulping efficacy. Therefore, aspen wood chips were treated with the white-rot fungus *C. subvermispora* for 4 weeks and then refined through a single-disk mechanical refiner. Pulp obtained from the fungus-treated chips had extensively fibrillated fibers that stained a deep orange with Simons stain (Fig. 4). In contrast, pulp obtained from the untreated control chips exhibited little fibrillation and stained a deep blue (Fig. 4). This showed that fibers obtained from the fungus-treated chips could be differentiated from those obtained from control chips based on the yellowing of fiber ends [80]. We

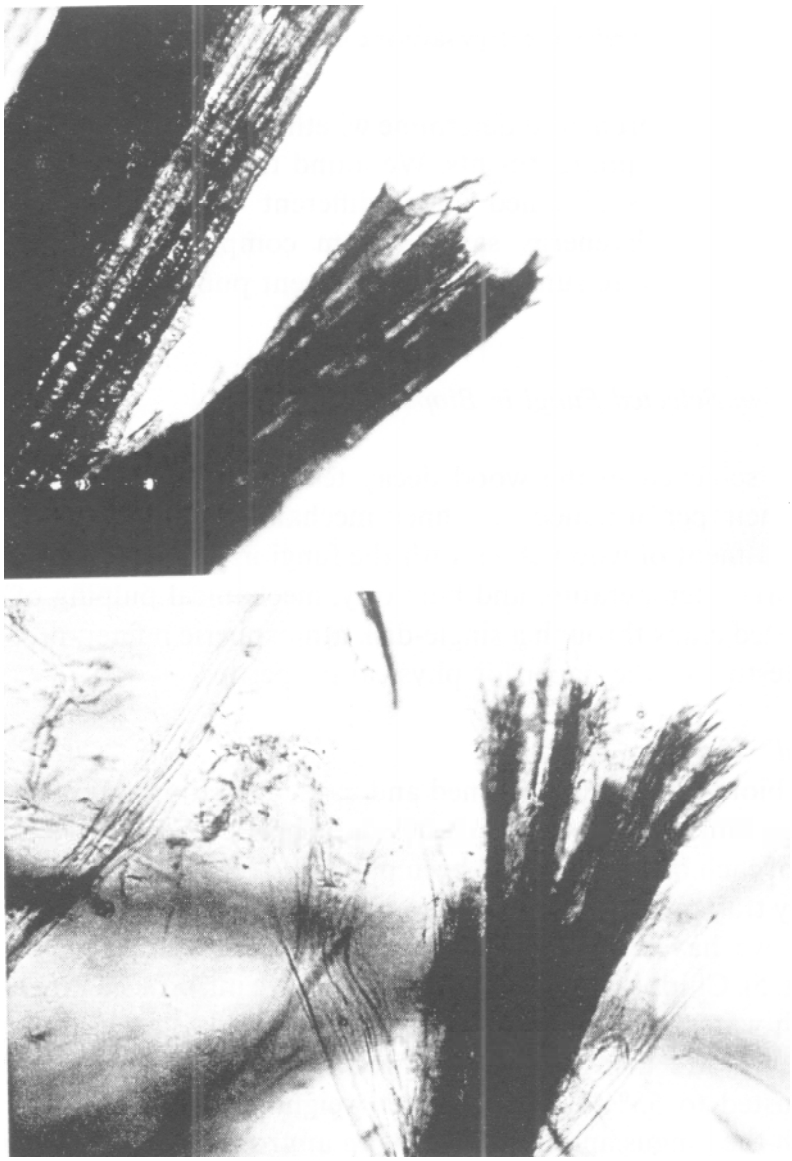

Fig. 4. Simons staining of control *(top)* and biopulp *(bottom).* Pulps from untreated control wood chips and *Ceriporiopsis subvermispora* - treated chips (4-week incubation) were passed through the refiner only once (Canadian Standard Freeness of pulps at about 700 ml) and then stained [80]

Table 5. Correlation of yellowing of fiber ends with energy savings using selected *Ceriporiopsis* spp. on aspen wood chips (2-week incubation)

Species/strain	Yellowing of fiber ends (%)			
	None	Slight	Intermediate	Advanced
Control	+			
C. rivulosa L-10602-sp.	+ (0)			
C. rivulosa PiiRTO-26K225	+ [a]	+ [a] (3%)[b]		
C. pannocincta FP-101181-sp.	+	+ [a] (4%)		
C. subversmispora L-3292		+	+ (7%)	
C. subvermispora CZ-3			+ (12%)	
C. subvermispora L-9186-sp.			+	+ (16%)

[a] Two plus signs for one treatment indicate that the staining pattern was in-between the two categories

[b] Values in parentheses represent refining energy savings compared to that of the untreated control

further evaluated this approach to determine whether it can be used to monitor differences among fungal pretreatments. We found that the intensity of yellow staining of biopulp fibers obtained under different experimental conditions correlates very well with energy savings from completed biopulping runs (Table 5). These results were summarized in a recent publication [81].

2.3.2 Evaluation of Selected Fungi in Biopulping Runs

White-rot fungi screened in the wood decay test or with Simons stain were evaluated for their performance in refiner mechanical pulping. The process involved the treatment of wood chips with the fungi in bioreactors on a bench scale at appropriate temperature and humidity, mechanical pulping of control and fungus-treated chips through a single-disk atmospheric refiner, preparation of paper, and testing of the paper for physical properties.

2.3.2.1 Method

Three types of bioreactors were designed and used: a rotating drum bioreactor, a stationary tray bioreactor, and an aerated static bed bioreactor. Details of the configuration of each bioreactor have been published (rotating drum bioreactor [82], stationary tray bioreactor [83], and aerated static bed bioreactor [84]). In recent studies, we have used the simple and inexpensive aerated static bed bioreactor (Fig. 5). Chips (1500 g, dry weight basis) are introduced into each 21-1 bioreactor with water (containing nutrients [85] and additive, if any), and the loaded bioreactors are then usually sterilized by autoclaving. Chip moisture content is adjusted to 55%-60% on a wet weight basis. The chips are then inoculated with the fungus and incubated at an appropriate temperature (39°C for *P. chrysosporium* and 27°C for other fungi) for 2-4 weeks (in most cases 2 weeks) with humidified air ($0.022711^{-1}min^{-1}$). Details are described in previous publications [83, 84, 86].

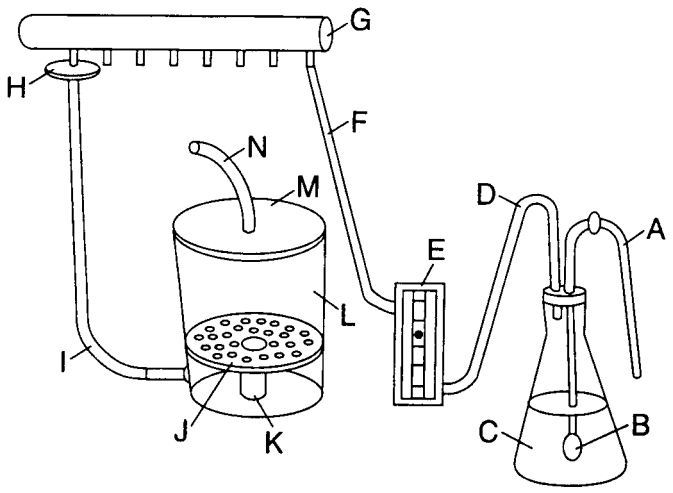

Fig. 5. Diagram of a static bed bioreactor [84]. The bioreactor was fabricated from a polypropylene vessel *(L).* The top of the vessel is sealed with a lid *(M),* which is vented to the atmosphere through an exit tube *(N).* Suspended above the bottom of the reactor *(L)* is a perforated polypropylene floor *(J),* supported by a stand *(K).* Air for the bioreactor comes from a regulated supply and passes through tubing *(A)* to a fritted glass gas dispenser *(B)* in a humidification flask *(C)* containing sterile water. Humidified air passes through tubing *(D)* to a rotameter *(E)* and through tubing *(F)* to a manifold *(G).* Humidified air from manifold *(G)* is passed through a filter *(H)* before passing through tubing *(I)* to the reactor *(L)* base

After harvest, the untreated control chips and the fungus-treated chips are fiberized by multiple passes through a Sprout–Waldron Model D 2202 single rotating 300-mm-diameter disk atmospheric refiner. Energy consumed during fiberization and subsequent refining is measured using an Ohio Semitonic Model WH 30-11195 integrating watt-meter attached to the power supply side of the 44.8-kW electric motor. Pulps are then refined to Canadian Standard Freeness (CSF) values just above and just below 100 ml. CSF is an arbitrary measure of the rate of water drainage from a pulp slurry. Handsheets (60 g m^{-2}) are made with these two pulp samples and tested for physical properties using Standard TAPPI methods. Energy values and physical properties are regressed to 100 ml CSF to facilitate comparison. Details of energy measurements, handsheet preparation, and testing methods have been described [72, 82,87, 88].

2.3.2.2 Energy Savings and Physical Properties

In early experiments, several white-rot fungi screened initially by the wood decay test were further screened for their biomechanical pulping performance using aspen (hardwood) wood chips [72, 73, 82, 88]. Based on energy savings and paper strength improvements, six fungi were initially selected: *P. chrysosporium, P. tremellosa, P. subserialis, P. brevispora, D. squalens,* and *Poria medulla-panis.* Of these, more emphasis was given to *P. chrysosporium* because it is by far the most studied white-rot fungus, grows rapidly, and competes well with indigenous microorganisms of wood chips. Periodically, additional fungi

Table 6. Energy savings and changes in physical properties from biomechanical pulping of loblolly pine chips with different white-rot fungi (4-week incubation)

		Strength properties			Optical properties		
Fungus	Energy savings (%)	Burst index ($kN\ g^{-1}$)	Tear index ($mNm^2\ g^{-1}$)	Tensile index ($Nm\ g^{-1}$)	Brightness (%)	Opacity (%)	Scattering coefficient ($m^2 k\ g^{-1}$)
Pc	14	14	1	19	– 27	– 2.3	– 29.8
Hs	26	12	32	– 12	– 21	– 3.6	– 27.6
Pb	28	– 4	21	– 9	– 24	– 2.9	– 28.1
Ps	32	– 29	9	– 36	– 28	– 3.7	– 34.6
Pt	36	– 18	12	– 17	– 24	– 4.5	– 32.6
Cs	42	32	67	– 1	– 22	– 4.4	– 30.9

PC = *Phanerochaete chrysosporium;* Hs = *Hyphodontia setulosa;* Pb = *Phlebia brevispora;* Ps = *Phlebia subserialis;* Pt = *Phlebia tremellosa; Cs = Ceriporiopsis subvermispora*

Table 7. Energy savings and changes in physical properties from biomechanical pulping of aspen wood chips with three strains of *C. subvermispora* (4-week incubation)

		Strength properties			Optical properties		
Strain	Energy savings (%)	Burst index ($kN\ g^{-1}$)	Tear index ($mN\ m^2 g^{-1}$)	Tensile index ($N\ mg^{-1}$)	Brightness (%)	Opacity (%)	Scattering coefficient ($m^2 k\ g^{-1}$)
FP-90031-sp.	40	23	131	17	– 18	– 2.0	– 33.8
L-6133-sp.	44	27	137	20	– 21	– 1.3	– 34.0
CZ-3	48	40	162	27	– 21	– 1.3	– 37.1

Table 8. Energy savings and changes in physical properties from biomechanical pulping of loblolly pine chips with three strains of *C. subvermispora* (4-week incubation)

		Strength properties			Optical properties		
Strain	Energy savings (%)	Burst index ($kN\ g^{-1}$)	Tear index ($mN\ m^2 g^{-1}$)	Tensile index ($N\ mg^{-1}$)	Brightness (%)	Opacity (%)	Scattering coefficient ($m^2 k\ g^{-1}$)
FP-90031-sp.	37	41	54	4	– 21	– 0.94	– 27.2
L-14807-sp.	30	44	59	3	– 19	– 0.63	– 24.9
FP-104027-T	21	45	47	11	– 21	0.0	– 23.1

identified for lignin selectivity on the basis of the wood decay test were also evaluated for their biomechanical pulping efficacy.

As the research progressed, emphasis was given to screening the fungi on loblolly pine (softwood) chips because this wood, together with other southern pines, is a major wood source for mechanical pulp mills in the United States. Some of the fungi selected as being best on aspen wood chips were evaluated on loblolly pine chips. Some of these results are presented in Table 6. All the fungi saved energy and some improved paper strength, but all adversely affected

paper optical properties. Based on energy savings and strength improvements, *C. subvermispora* was identified as the best fungus [86]. Different strains of this fungus were found to be effective on both aspen (Table 7) and loblolly pine (Table 8) [84]. A US patent was issued on the use of *C. subvermispora* for biomechanical pulping [89].

2.3.3 Optimization of Biopulping Runs

For biopulping, like any industrial microbial process, there is great opportunity to increase the effectiveness and efficiency and decrease the cost of the process through optimizing the variables. Our initial work was governed by "best guesses" for optimal biopulping conditions based on the literature, knowledge of fungal growth, and past experience. Initial research focused on the fungus *P. chrysosporium* and aspen chips; later, *C. subvermispora* became the focus of our study. Some of the initial results are in the following text.

2.3.3.1 Wood Batch, Chip Storage, and Chip Movement During Incubation

Some parameters, including wood batch and chip storage conditions (frozen, fresh, or dried at room temperature) did not seem to affect biopulping performance. In early experiments, fungus sensitivity to chip movement was observed when stationary and rotating drum bioreactors were used. Chip movement affected the extent of chip degradation, energy consumption during refining, and paper strength properties [72, 88]. Later studies, however, showed that shaking the aerated static bed bioreactors once a week during the 4-week incubation period had no appreciable effect on energy savings or paper strength properties.

2.3.3.2 Inoculum

In any industrial fermentation, the inoculum is of key importance. A number of variables affect the fermentation, including level, physical form, age, and viability. We examined some of these variables in a series of experiments.

The effect of different inoculum levels (2.5%, 5%, 10% and 20%, dry weight basis), using precolonized chips as inoculum, was studied with *P. chrysosporium* on aspen wood chips. The lowest inoculum level (2.5%) gave slightly lower energy savings than the other three levels.

We postulated that the addition of nutrient nitrogen to the inoculum would help build fungal biomass and vigor, which in turn would improve biopulping performance of *P. chrysosporium.* To test this hypothesis, two concentrations of glutamic acid (500 and 5000 ppm N) were added to the 5% wood chips inoculum prior to introducing the fungus. The results suggested that increased inoculum nitrogen had beneficial effects in terms of energy savings. However, the weight loss stimulated by the high nitrogen offsets these benefits.

Another approach that was tried to increase the inoculum vigor of *P. chrysosporium* was by increasing the age of the inoculum (2,4, and 6 weeks). We concluded that inoculum age has little influence under the conditions used.

In all of these experiments, precolonized wood chips were used as an inoculum. In other experiments, we evaluated a liquid inoculum (mycelial suspension) of *C. subvermispora* on aspen wood chips (0.1% on a dry wood basis). Results with the liquid inoculum were acceptable but not as good as the results with the chip inoculum. Because we did not measure the amount of fungus added with the colonized chips, we do not know whether the increased biopulping efficacy with chip inoculum was due to increased fungus content or an inherent advantage of delivering inoculum with wood chips.

2.3.3.3 Nutrients Requirement

The effect of nitrogen and carbon was studied with *P. chrysosporium* on aspen chips. Two levels each of nitrogen (25 or 250 ppm N as glutamic acid) and glucose (4000 or 40000 ppm) were added to bioreactors. The combination of 25 ppm nitrogen and 40000 ppm glucose produced the best results in terms of energy savings. Subsequently, the effect of another nitrogen source (ammonium tartrate) (0, 36, 108, and 324 ppm N) was examined. We found that 324 ppm N gave optimum results.

The use of a nonchemically defined nitrogen source, i.e., yeast extract, was also investigated with both *P. chrysosporium* and *C. subvermispora* on aspen wood chips. With *P. chrysosporium,* two levels of nitrogen (108 and 324 ppm) on a dry weight basis were tested with a uniform level of 40000 ppm lactose (dry weight basis). With *C. subvermispora,* three levels of nitrogen (108, 324 and 976 ppm) with a uniform level of 4000 ppm lactose (dry weight basis) were used. An energy saving of 32% with significant strength improvements was obtained after 2 weeks, one of the best results ever achieved with *P. chrysosporium.* With *C. subvermispora,* an energy saving of 52% with strength improvements was obtained for the highest nitrogen level (976 ppm); this result was better than average for this fungus on aspen chips.

In a few runs made without the addition of nutrients, we noted significant energy savings and paper strength improvements, although addition of nutrient nitrogen seemed to enhance the biopulping efficacy of these fungi.

2.3.3.4 Aeration

Solid-substrate fermentations are known to be affected markedly by aeration. In addition, the ligninolytic activity of fungi depends on oxygen availability. Consequently, we evaluated the influence of three air flow rates on the biopulping efficacy of *P. chrysosporium* on aspen chips: low ($0.001\ 1^{-1}\ min^{-1}$), medium ($0.022\ 1^{-1}\ min^{-1}$), and high ($0.100\ 1^{-1}\ min^{-1}$). The low flow rate was achieved using intermittent aeration. The medium and high flow rates gave comparable energy savings and had similar effects on strength properties. The low flow rate was suboptimal.

2.3.3.5 Wood Chip Sterilization

Our studies showed that *P. chrysosporium* could colonize unsterilized wood chips and perform biopulping when incubated in a bioreactor at its optimum

growth temperature (39°C), but not at suboptimal growth temperatures; *C. subvermispora* was found to be ineffective on unsterilized wood chips even at its optimum temperature (27–32°C).

2.3.3.6 Bleaching Studies

Fungal pretreatment reduces the brightness of the resulting mechanical pulps by as much as 15–20 Elrepho brightness points in 4 weeks and 8–10 Elrepho brightness points in 2 weeks. Consequently, the bleachability and brightness stability of aspen biorefiner mechanical pulps (BRMPs) were investigated. Comparisons were made with untreated aspen refiner mechanical pulp (RMP) and commercial mechanical pulps: chemithermomechanical pulp (CTMP), thermomechanical pulp (TM P), and groundwood (GW) pulp [90]. *P. chrysosporium* was used for the pretreatments reported here; subsequent experiments demonstrated that aspen chips treated with *C. subvermispora* respond similarly to bleaching.

Either alkaline hydrogen peroxide or sodium hydrosulfite readily increased the brightness of aspen BRMP (Table 9). Fungal pretreatment enhanced the bleachability of the aspen pulps examined. The BRMPs increased by more brightness points than did corresponding untreated pulps. However, because the initial brightness values of the BRMPs were lower than those of the corresponding untreated pulps, the bleached brightness values were not as high at a given chemical charge as those of untreated mechanical pulps. Aspen BRMP was readily bleached to 60% Elrepho brightness with 1% sodium hydrosulfite–a brightness suitable for newsprint; brightness values approaching 80% were achieved with a two-step bleach sequence. Thus, bleachability of biomechanical pulps appears not to be a serious problem.

Table 9. Bleaching results with aspen pulps (90)

Pulp	Chemical charge	Brightness (%)
GW	Unbleached	63.1
	1.5% H_2O_2	80.8
	1% $Na_2S_2O_4$	71.9
CTMP	Unbleached	62.0
	3% H_2O_2	78.3
	l% $Na_2S_2O_4$	66.3
TMP	Unbleached	60.2
	3% H_2O_2	78.6
	1% $Na_2S_2O_4$	66.9
RMP (control)	Unbleached	62.2
	3% H_2O_2	80.0
	1% $Na_2S_2O_4$	77.2
BRMP	Unbleached	51.8
	3% H_2O_2	76.0
	1% $Na_2S_2O_4$	59.3
	Two-step bleach 3% H_2O_2 1% $Na_2S_2O_4$	78.0

Table 10. Effluents from first refiner pass of control and fungus-treated aspen chips (91)

Treatment	BOD ($g\ kg^{-1}$ pulp)	COD ($g\ kg^{-1}$ pulp)	Toxicity ($100\ EC50^{-1}$)
Control	40	74	17
C. subvermispora	36	100	4

Brightness stability was evaluated by subjecting handsheets to accelerated thermal and photo-aging tests [90]. The stability of BRMP was slightly lower than that of RMP but slightly higher than that of CTMP.

2.3.3.7 Analysis of Effluents

Samples of the wastewater from the first refiner passes of aspen chips pretreated with either *C. subvermispora* or *P. chrysosporium* were analyzed for biochemical oxygen demand (BOD), chemical oxygen demand (COD), and Microtox toxicity. Fungal pretreatment of aspen chips with either *P. chrysosporium* or *C. subvermispora* during biopulping substantially decreased effluent toxicity (Table 10). The BOD values for effluents from fungus-treated pulps were either slightly lower or higher than those of RMPs, depending upon the fungal species and whether nutrients were used. The COD values for effluents from fungus-treated pulps were considerably higher than those from RMP runs, probably because of the release of lignin-related products. Based on these data, we conclude that the effluent load from the biopulping runs should be lower and more benign than that of commercial CTMP mills [91].

2.3.3.8 Global Analysis of Data

The data from all runs, regardless of experimental conditions, were analyzed statistically in a global manner. The data were plotted as notched box plots [92] so that any statistically significant differences (± 0.95 level) between the untreated control and biopulping runs could be observed. The global analysis greatly simplified the overall interpretation of a large amount of data. To illustrate the analysis, a comparison of the efficacy of fungal species in terms of refiner energy consumption in the biopulping of aspen chips is shown in Fig. 6. In subsequent analysis, *P. chrysosporium* was found to be less effective than *C. subvermispora* on aspen wood chips and relatively ineffective on loblolly pine chips. The energy required for refining was reduced, in many cases by 40% or more (e.g., Fig. 6) with little loss of wood substance (< 10%), except when chemically defined, high nutrient nitrogen supplements were used. Handsheet density was decreased somewhat by treatment of aspen with *P. chrysosporim,* and more with *C. subvermispora;* with pine, density was unaffected (Fig. 7).

We treated *P. chrysosporium* / aspen, *C. subvermispora* / aspen, and *C. subvermispora* / pine as three separate sets of data, with all runs of these combinations

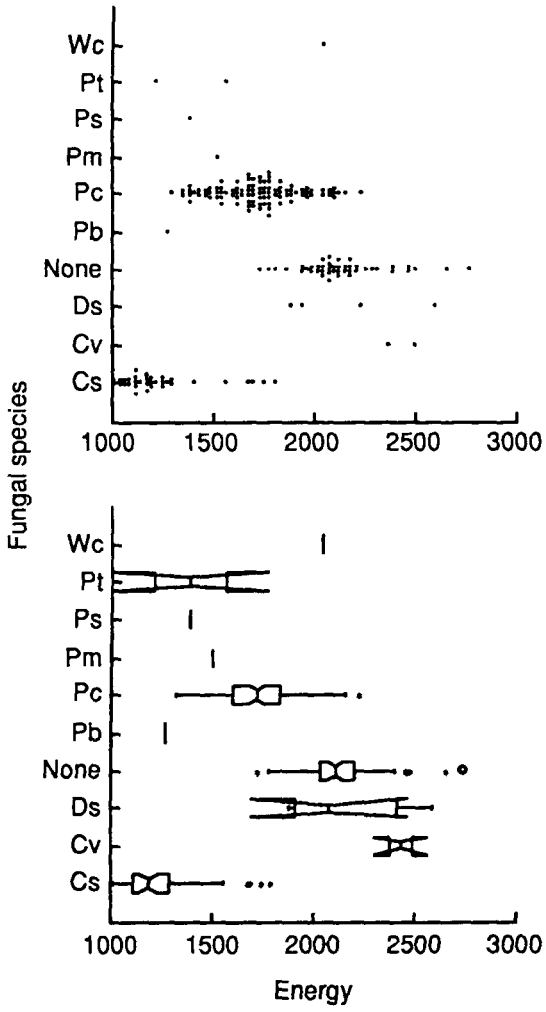

Fig. 6. Refiner energy consumption of aspen chips treated with different fungi *(top* dot-plot, *bottom* box plot) [52]. *Wc Wolfiporia cocos* (a brown-rot fungus), *Pt Phlebia tremellosa, Ps Phlebia subserialis, Pm Pholiota mutabilis, Pc Phanerochaete chrysosporium, Pb Phiebia brevispora, Ds Dichomitus squalens, Cv Coriolus (Trametes) versicolor, Cs Ceriporiopsis subvermispora* (all white-rot fungi)

included. Chip pretreatment with either fungus significantly increased burst index of handsheets from aspen but not that of handsheets from pine (Fig. 8). Chip pretreatment with either fungus also significantly increased tear index (single-ply test). Only *C. subvermispora* was examined with pine; tear index increased significantly (Fig. 9). Tensile index was increased significantly by chip pretreatment with either fungus in the case of aspen, but not pine (Fig. 10). Handsheet brightness was significantly decreased by pretreating chips of either wood with either fungus (Fig. 11), as was scattering coefficient with *C. subvermispora* on both woods and *P. chrysosporium* on aspen (data not shown); opacity was not significantly affected by either fungus (data not shown).

2.3.4 Microscopy Studies

To gain insight into the mechanism of biopulping, we examined at the microscope level the growth patterns of the fungi in wood, the effects of fungi on wood cell walls, and the appearance of handsheets made from biopulps.

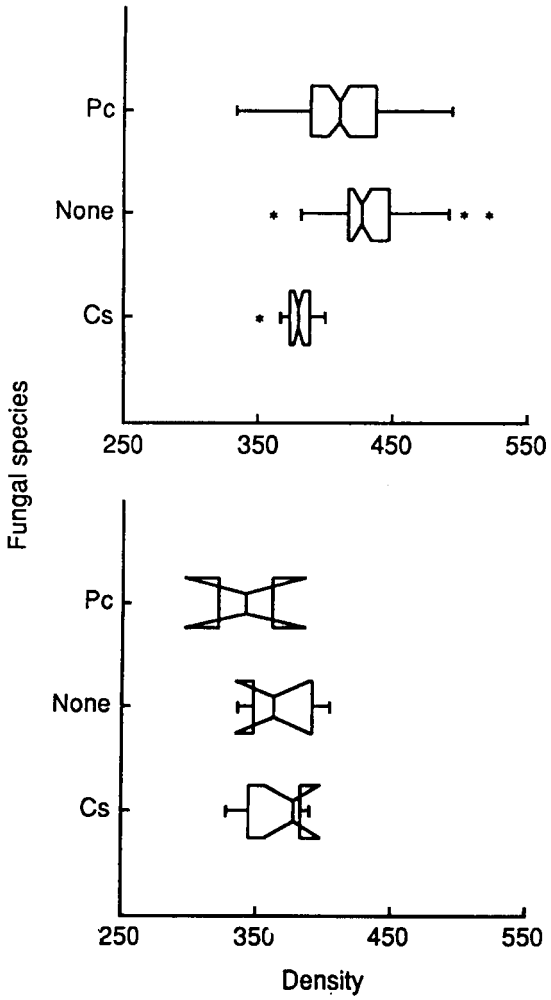

Fig. 7. Density of handsheets made from aspen *(top)* and loblolly *(bottom)* pine chips treated with *Phanerochaete chrysosporium (Pc)* or *Ceriporiopsis subuermispora (Cs)* [52]

2.3.4.1 Fungal Growth Patterns

Scanning electron microscopy was used to observe fungal growth in and degradation of nutrient-supplemented aspen chips after a 3-week treatment with *P. chrysosporium.* The fungus grew well both across the chip surfaces (Fig. 12) and throughout the cell walls. The hyphae penetrated the chips through the lumina of wood vessels and fiber cells as well as through natural wood cell pits and fungal boreholes. Partial degradation of the cell lumen walls was evident. Erosion troughs and localized wall fragmentation or thinning were clearly visible as was a generalized swelling and relaxing of the normally rigid wood cell wall structure (Fig. 13). Aspen wood chips treated with *C. subvermispora* showed packed hyphae within the ray cells. Many crystals of calcium oxalate were found on the hyphae (Fig. 14) during both the incipient and the advanced stages of growth. Our observations suggest that the physical basis for the biopulping efficacy of the fungal treatment is likely to involve an overall softening and swelling of the wood cell walls as well as thinning and fragmentation in localized areas [93].

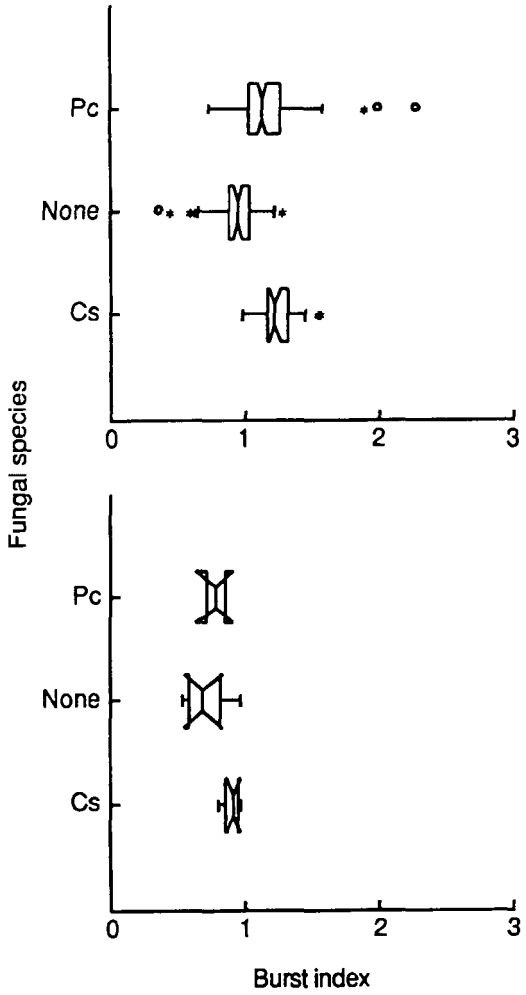

Fig. 8. Burst index for handsheets made from aspen *(top)* and loblolly pine *(bottom)* chips treated with *Phanerochaete chrysosporium (Pc)* or *Ceriporiopsis subvermispora (Cs)* [52]

2.3.4.2 Fibers

In another investigation, we compared the microscopic appearance of BRMP with that of RMP, GW pulp, TMP, CTMP, neutral sulfite semichemical pulp (NSSC), and kraft pulp. When fiberized, BRMP emerged wool-like, looser, and bulkier, with fibers rather uniform in length; the pulp also exhibited abundant fibrillation. In contrast to BRMP, RMP fibers were not as wide, appeared to be stiffer, were of different lengths, and had only moderate fibrillation. The GW pulp fibers were stiff and of various lengths, showed reduced fibrillation, and were accompanied by debris. The TMP and CTMP fibers appeared stiff and were of various lengths, although longer than RMP fibers, and had moderate fibrillation. The TMP and CTMP fibers were more twisted than the BRMP fibers. The NSSC pulp exhibited few stiff fibers and the fibers were of various lengths; they appeared to be more compressible and conformable when compared to the mechanical pulp fibers. Compared to BRMP, NSSC pulp fibers were not as compressed and were more variable in length. Kraft pulping

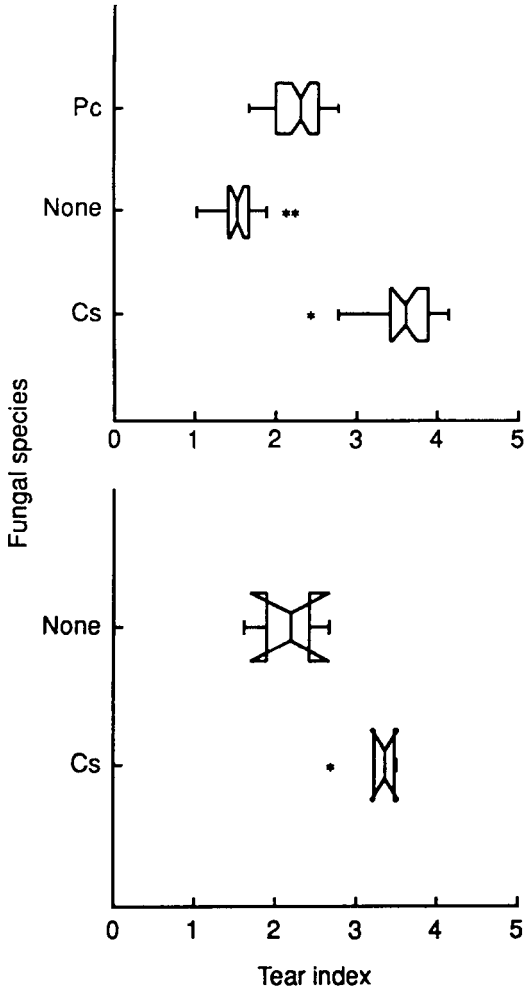

Fig. 9. Tear index (single-ply tear) for handsheets made from aspen *(top)* and loblolly pine *(bottom)* chips treated with *Phanerochaete chrysosporium (Pc)* or *Ceriporiopsis subvermispora (Cs)* [52]

produced more uniform, separated, and collapsed fibers, with abundant fibrillation. The BRMP appeared to be similar to the kraft pulp. These results were summarized by Sachs et al. [79, 94].

2.3.4.3 Handsheets

Aspen BRMP produced a stronger handsheet than did aspen TMP and GW pulp. Aspen NSSC pulps appeared to be superior to aspen BRMP in handsheet properties. Of all the pulps, aspen kraft pulp had the highest sheet strength properties. To gain insight and visually assess how the fiber morphology in these pulps may have contributed to sheet strength properties, we examined cross sections of handsheets. Handsheets made from mechanically processed pulps showed uncollapsed fibers, leading to poor conformability and reduced bonding. The NSSC and kraft pulps gave handsheets that exhibited fibers of enhanced compressibility and conformability. Handsheets prepared from BRMP visually resembled the kraft handsheets, exhibiting good compressibility and conformability of the fibers [79, 94].

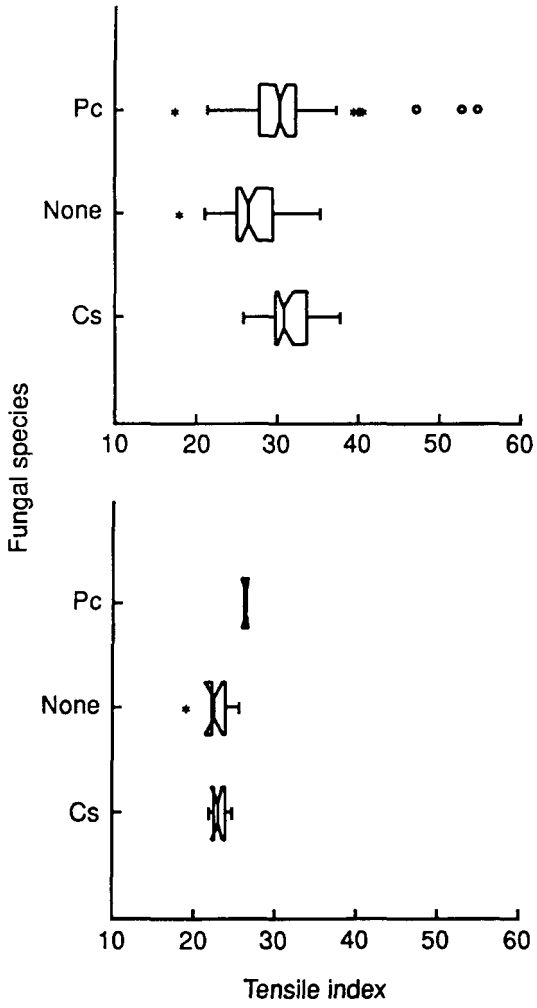

Fig. 10. Tensile index for handsheets made from aspen *(top)* and loblolly pine *(bottom)* chips treated with *Phanerochaete chrysosporium (Pc)* or *Ceriporiopsis subvermispora (Cs)* [52]

2.3.5 Engineering and Economic Studies

The engineering and economics studies related to biopulping scale-up are summarized in the PhD thesis of Wall [95]. The goal of these studies was to develop the basic scientific and engineering knowledge needed for the commercial utilization of fungi in chip piles. We investigated process scale-up by obtaining kinetic data, developing process models, and implementing the processes on a pilot scale [96]. Initial studies were performed using *P. chrysosporium* on aspen wood chips.

Mathematical modeling of biopulping was done to assess the factors that might kinetically limit fungal growth. Assumptions concerning the stoichiometry of biological wood degradation were used to predict important parameters needed to model the growth of *P. chrysosporium* in wood chips. Next, the effects of mass transfer on the growth of *P. chrysosporium* under typical biopulping conditions were considered. Finally, mass and energy balances were modeled for an isolated plug of wood chips, and the resulting system of differential equations was integrated numerically.

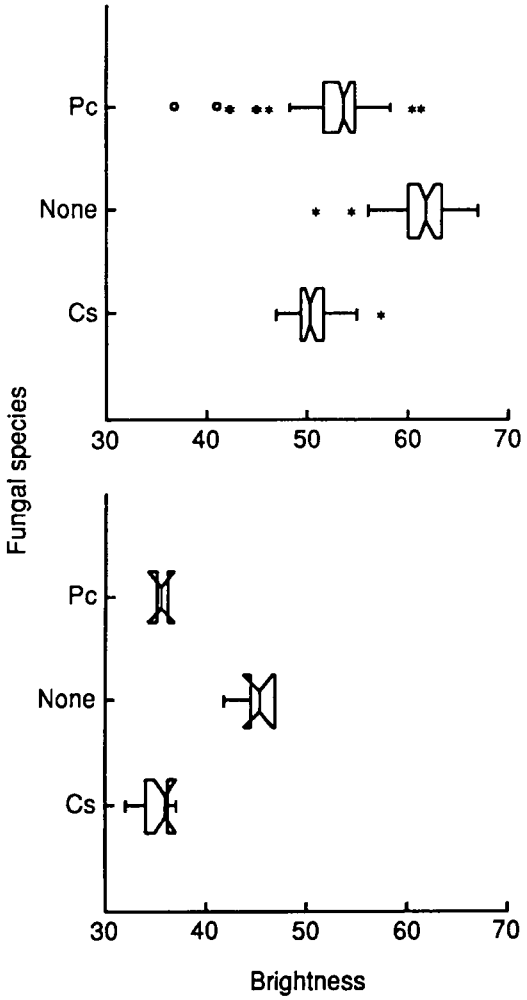

Fig. 11. Brightness of handsheets made from aspen *(top)* and loblolly pine *(bottom)* chips treated with *Phanerochaete chrysosporium (Pc)* or *Ceriporiopsis subvermispora (Cs)* [52]

Based on the kinetic and modeling studies, two bioreactor designs were considered: a packed-bed reactor and a chip-pile-based system (Fig. 15). The packed-bed bioreactor envisaged for the biopulping process is a bed of wood chips with controlled air flow. Packed-bed reactors allow better control of process conditions. The drawbacks to these reactors are that they require much greater capital expenditure and entail higher operating costs than chip-pile based systems. A chip-pile based system is defined here as an industrial-size chip pile modified to increase temperature and moisture control. The advantage of the chip pile is reduced cost as compared to that of a packed-bed reactor; the disadvantage is reduced process control.

Early in the biopulping research, Harpole et al. [97] conducted an economic evaluation based on a thermomechanical process model. Results indicated that a 25% reduction in pulping energy by fungal treatment would save $21 (US) per air dry ton (adt) of pulp ($33 with 40% energy savings). The capitalized value of the energy savings was estimated to be about $250000 for each percentage of energy saved, at an electricity cost of $0.035/kWh. Thus, a sizeable capital

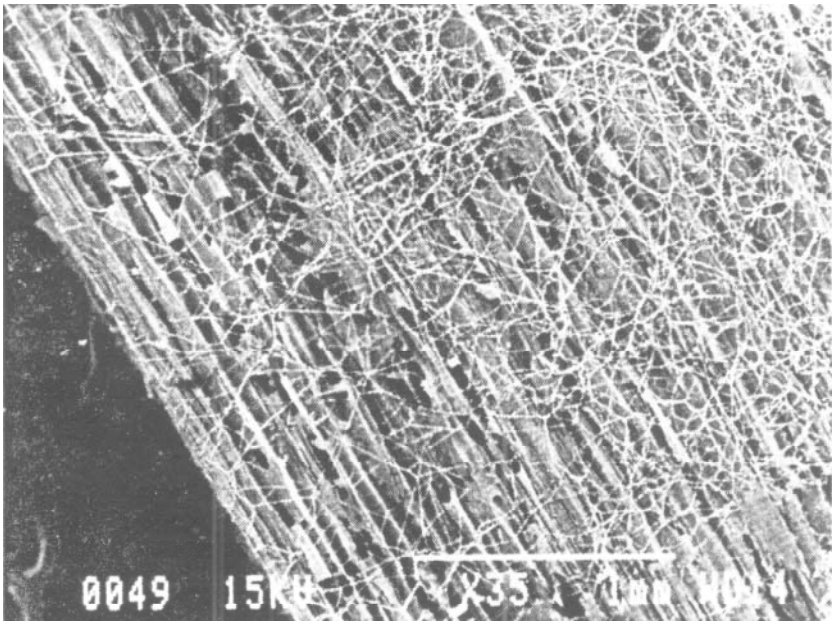

Fig. 12. Web-like hyphal network on surface of nutrient-supplemented aspen wood chip during 3-week treatment by *Phanerochaete chrysosporium* [93]. *Bar* = 1 mm

expenditure for the biotreatment could be accommodated. More recently, an economic model based on mass and energy balances was made for a controlled packed-bed reactor process and a chip-pile-based system. The controlled reactor process yields a pretax return on investment (ROI) of 21%, whereas the chip-pile-based system shows a pretax ROI of 106%–217% (depending on the inoculum costs). The details are summarized in the following text.

2.3.5.1 Economic Analysis of Packed-Bed Reactor

The capital and operating costs were estimated for a 300 metric ton/day biological pretreatment using *P. chrysosporium* on aspen and assuming a treatment time of 2 weeks and a dry weight loss of 5%. The air flow rate was assumed to be 0.59 VVM. The operating costs considered were steam, inoculum, and electricity for aeration. A reasonable figure for inoculum costs was chosen based on the information concerning a similar commercial process (see Section 2.3.6). The most optimistic scenario yields a pretax ROI of 21% (Table 11, Case 1).

2.3.5.2 Economic Analysis of Chip-Pile-Based System

A chip-pile-based system with ductwork to provide aeration, piping to provide steam, and a sprayer to apply inoculum to chips on a conveyor was chosen for this analysis. The aeration rate and inoculum level were the same as those used for the packed-bed reactor; therefore, the operating costs for a chip-pile-based system are the same as those given for the packed-bed reactor. The capital costs

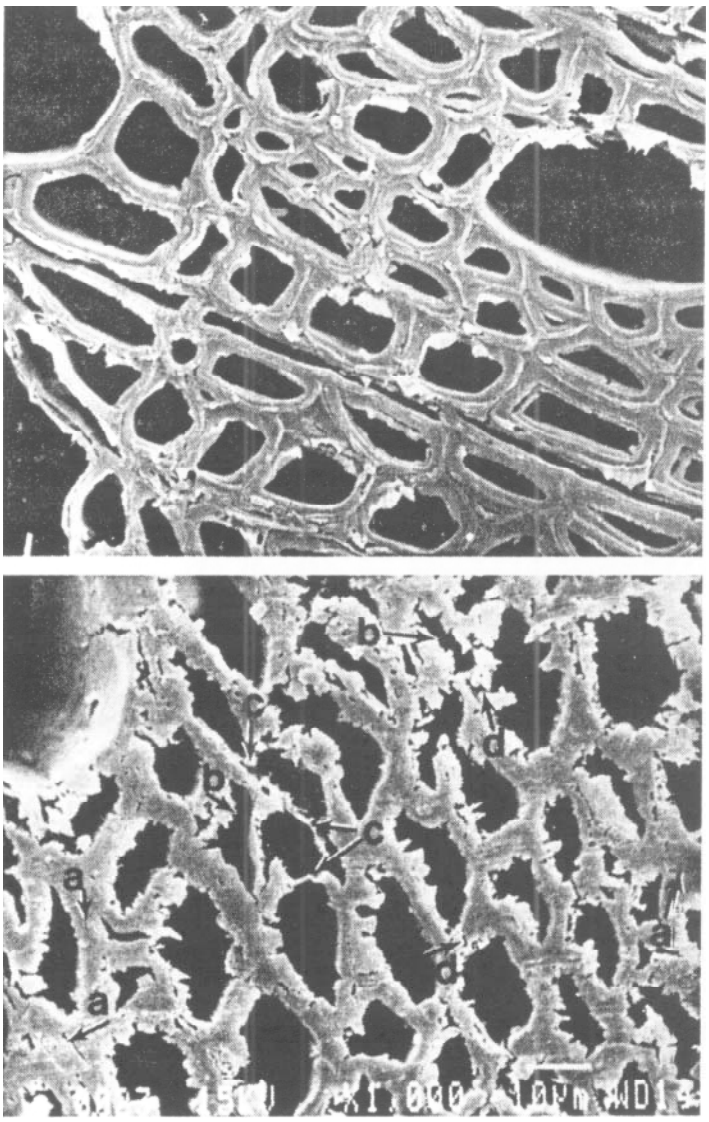

Fig. 13. The normally rigid cell wall structure within aspen chips *(top)* was modified *(bottom)* by 3-week treatment with *Phanerochaete chrysosporium* [93]. Modifications included cell wall swelling *(a)*, enzymatic softening or relaxing resulting in the partial collapse of the tube-like structure *(b)*, and localized areas of wall thinning *(c)* or fragmentation *(d)*. *Bar* = 10 μm

considered were those for fans, ductwork, inoculum tanks, and humidification. The analysis showed a pretax ROI of 106%–217%, depending on the inoculum costs (Table 12).

Our recent work has focused on *C. subvermispora* and loblolly pine, with special attention to those factors most likely to affect the economics of a chip-pile-based system. These factors are achieving the necessary degree of asepsis, lowering the cost of fungal inoculum, and maintaining a hospitable environment in the chip pile.

2.3.5.2.1 Asepsis. In early work, the beneficial effects of biopulping on a laboratory scale were seen when wood chips were sterilized by autoclaving. *C. subvermispora* was not found to be aggressive enough to compete with indigenous microorganisms in unsterilized wood chips. Recently, we discovered that

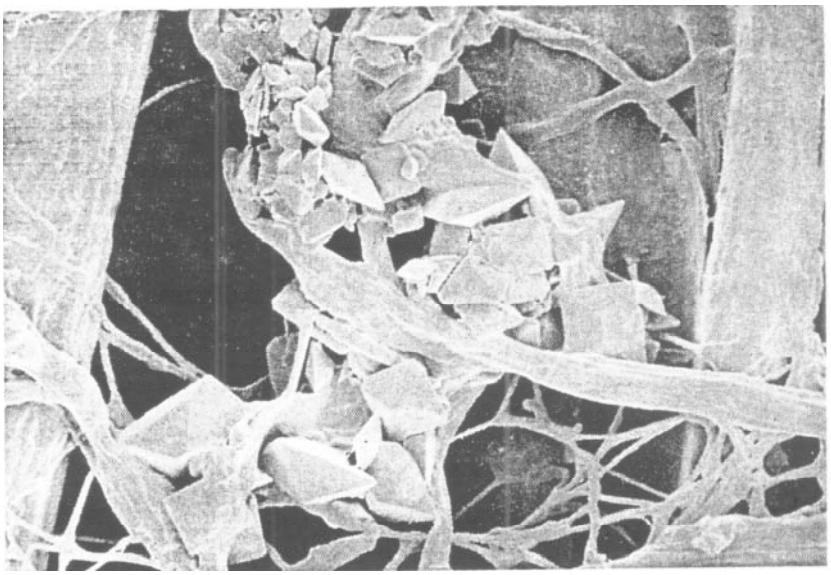

Fig. 14. Calcium crystals on the surface of hyphae of *Ceriporiopsis subvermispora* on aspen wood chips (4-week treatment). 1 cm = 5 μm

a brief atmospheric steaming of the chips allowed *C. subvermispora* to colonize and be effective [98]. This condition is now being studied from an engineering standpoint.

2.3.5.2.2 Fungal Inoculurn. One of the major costs foreseen during the scale-up of biopulping is inoculum production. Therefore, several experiments were aimed at determining the best inoculum level of *C. subuermispora* for saving energy and improving paper strength properties in a 2-week incubation. We found that 0.3% inoculum (dry weight basis) saved 19% energy and improved paper strength properties, such as tear index, significantly compared to the control (Table 13). This amount of inoculum is quite high. However, we discovered that the amount of inoculum can be lowered to 0.0005% (dry weight basis) or less by adding a cheap and commercially available nutrient source, corn steep liquor, to the mycelial suspension. This low amount of inoculum is now well within a commercial range. Subsequent studies have also identified better strains of *C. subvermispora* that yield up to 38% energy savings and improve tear index by 51% [98, 99]. Other nonchemically defined additives, including yeast extract and molasses, have shown promise in biopulping, but they have not been found to be as effective as corn steep liquor.

2.3.5.2.3 Chip Pile Environment. Instrumented bioreactors were constructed and are now in use to mimic chip piles. Current research is aimed at methods for controlling temperature, aeration, and moisture in chip piles so that the fungal treatment is effective. These data would support the design of a chip-pile-based system.

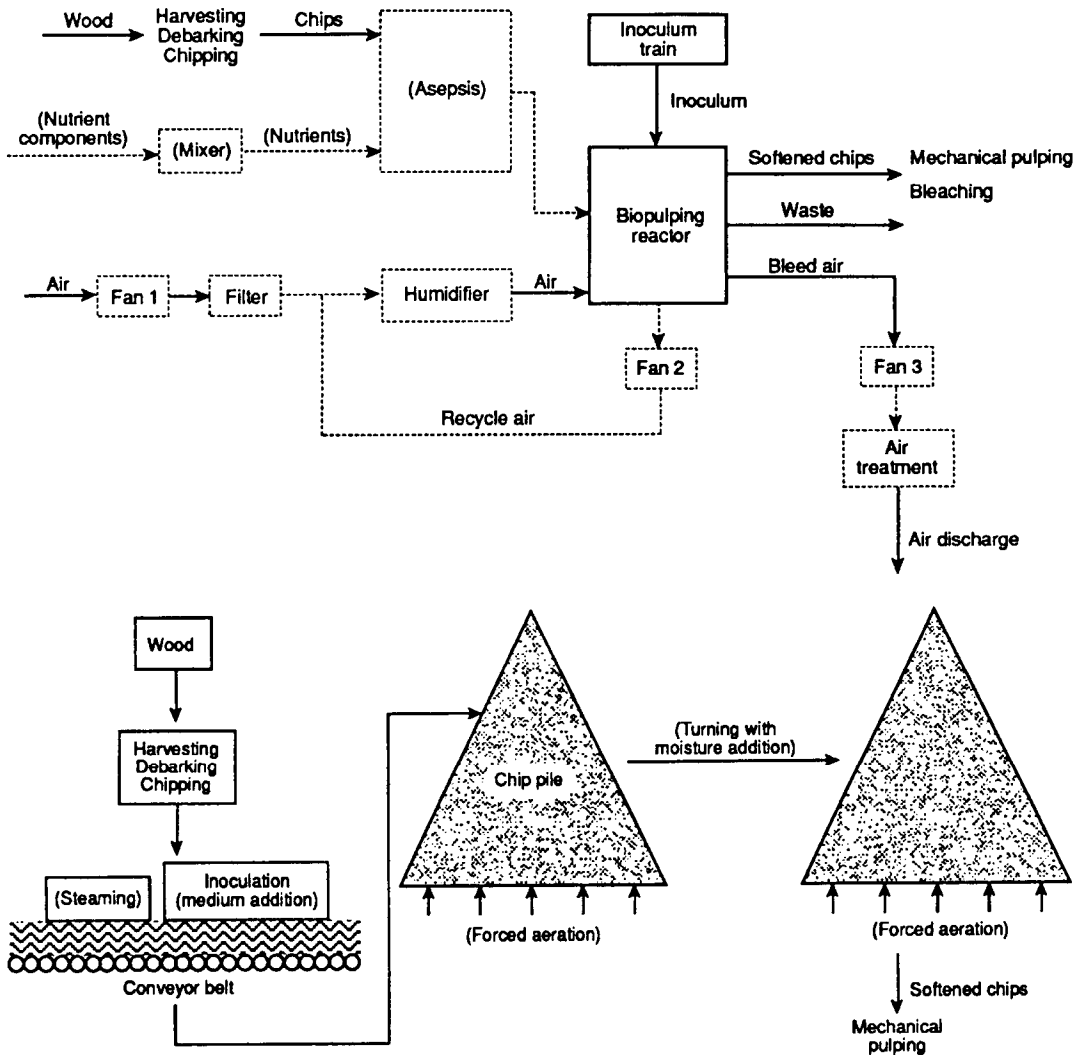

Fig. 15. Flowsheets for packed-bed reactor *(top)* and chip-pile-basedsystem *(bottom)*. Operations in parentheses are optional [95]

2.3.6 *Prospects for Biopulping Commercialization*

During the course of the biopulping research, one of the sponsoring companies developed and commercialized a biotechnological process that is similar to biopulping in many respects, and it deserves mention here. Cartapip is an industrial fungal inoculum of *(Ophiostoma piliferum* which was developed by Sandoz Chemicals Biotech Research Corporation (now Clariant Biotech Research Corporation) and is marketed by the US Sandoz Chemicals Corporation (now Clariant Corporation). The fungus is a naturally occurring and ubiquitous "blue stain" organism. It is nonpathogenic. A dilute slurry of the product (a powder), consisting of fungal spores, is sprayed onto wood chips as they are piled for storage prior to pulping. The spores germinate and the fungus grows aggressively in the chip piles. Within the wood chips, the fungus grows mainly in ray

Table 11. Economic feasibility of a packed-bed reactor [95]

	Case 1	Case 2	Case 3
Installed equipment costs	$5000000	$10000000	$17000000
Working capital	$206750	$206750	$206750
Total capital investment	$5206750	$10206750	$17206750
Utility costs	$2.46	$2.46	S2.46
Inoculum costs	$3.00	$3.00	$3.00
Labor	$0.76	$0.76	$0.76
Yield losses	$2.46	$2.46	$2.46
Depreciation	$4.96	$9.72	$16.39
Total operating cost	$13.64	$18.40	$25.07
Pretreatment value	$23.49	$23.49	$23.49
Gross profit	$9.85	$5.09	– $1.57
Pretax ROI	21%	5%	– l %

Table 12. Economic feasibility of a chip-pile-based reactor [95]

	Case 1	Case 2	Case 3
Installed equipment costs	$500000	$500000	$500000
Working capital	$206750	$206750	$206750
Total capital investment	$706750	$706750	$706750
Utility costs	$2.46	$2.46	$2.46
Inoculum costs	$3.00	$5.00	$10.00
Labor	$0.76	$0.76	$0.76
Yield losses	$2.46	$2.46	$2.46
Depreciation	$0.76	$0.76	$0.76
Total operating cost	$9.35	$11.35	$16.35
Pretreatment value	$23.49	$23.49	$23.49
Gross profit	$14.14	$12.14	$7.14
Pretax ROI	217%	180%	106%

Table 13. Energy savings and strength properties during biomechanical pulping of loblolly pine chips with *C. subvermispora* (2-week incubation)

Treatment (% inoculum on dry weight basis)	Energy savings[a] (%)	Strength properties	
		Burst index (k N g^{-1})	Tear index (m N $m^2 g^{-1}$)
Control	—	0.62 ± 0.05[b]	1.67 ± 0.13
0.01	4	0.63 ± 0.04	1.89 ± 0.09
0.05	11	0.71 ± 0.04	2.16 ± 0.20
0.10	12	0.74 ± 0.03	2.13 ± 0.14
0.15	12	0.70 ± 0.06	2.04 ± 0.15
0.30	19	0.70 ± 0.05	2.14 ± 0.15

[a] Energy savings are calculated on the basis of untreated control values
[b] Standard deviation

parenchyma cells and resin ducts [100], where its distinctive activity is to degrade extractives within about 2 weeks during storage [101]. It is not capable of degrading lignin or cellulose, but it degrades hemicelluloses to a very small extent. Partial removal of extractives helps solve several problems associated with pitch, including downtime for cleaning equipment, breakage of paper on the paper machine, decreased paper strength, and holes in the paper caused by sticky spots on rolls [102].

The Cartapip process has shown other beneficial effects as well. It improves incoming chip quality by inhibiting the growth of dark-colored fungi [103, 104]. This in turn increases chip and pulp brightness, and reduces the need for bleaching chemicals. The Cartapip process also prevents wood losses caused by wood-degrading microorganisms. During chemical pulping, the process increases yields and reduces rejects because of the improved penetration of pulping liquors through empty resin canals and ducts of wood chips [105].

The development of the Cartapip process shows that biological treatment of wood chips can be successfully implemented on an industrial scale. At this point in the investigations, it would appear that biopulping has a good chance of commercial success. Four recent developments have led to this optimism: (1) the discovery of *C. subvermispora*, which is effective on both hardwood and softwood species, (2) the finding that brief steaming can decontaminate the surfaces of wood chips so that *C. subvermispora* can take over, (3) the use of unsterilized corn steep liquor to dramatically reduce the inoculum quantity (from 3 kg to 0.25 g fungus per ton of wood [*dry wood basis*]), and (4) the demonstration of a successful l-ton chip pile (green wood) where the fungal pretreatment saved 32% electrical energy. A recently conducted 100-ton (green wood) outdoor chip pile experiment produced results similar to those obtained using laboratory scale bioreactors.

Our efforts so far have focused on the use of fungal treatments prior to refiner mechanical pulping. Recent studies in our laboratory and in others suggest that the fungal pretreatment is also effective for depitching [106] and that it gives benefits with thermomechanical pulping, chemithermomechanical pulping [107], sulfite pulping [106, 108, 109], and kraft pulping [110, 111].

Acknowledgements. We thank the following for their significant contributions to this research: Michael Attridge, Todd Burnes, Dough Cameron, Kory Cease, Leatha Dameron, Marilyn Effland, Mary Greenheck, George Harpole, Eric Horn, Jane Kohlman, John Koning Jr., Gary Leatham, Michael Lentz, Ed Lightfoot, Efrat Livney, Lou Lunte, Gary Myers, Lewis Otjen, Jean Pierick, Sanya Reyes-Chapman, Irv Sachs, Gary Scott, Jane Sherwood, Marguerite Sykes, Freya Tan, Ted Wegner, and Mary Beth Wall. This research was partially funded by the following companies: Andritz Sprout-Bauer, Boise-Cascade Corporation, Cellulosa Puerto Piray S. A., Champion International Corporation, Chimica del Friuli, Consolidated Papers Incorporated, The Dow Chemical Company, Great Northern Nekoosa Corporation (Georgia Pacific), James River Corporation, Leykam Mürzataler AG, Mead Corporation, Metsä Serla Oy, Nelco Chemical Company, Novo Nordisk A/S, Potlatch Corporation, Procter and Gamble Cellulose Company, Clariant Corporation, Scott Worldwide, Union Camp Corporation, and Weyerhaeuser Paper Company.

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