Fungi in lignocellulose breakdown and biopulping

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Biological pulping has the potential to improve the quality of pulp, properties of paper and to reduce energy costs and environmental impact relative to traditional pulping operations. It has been suggested that energy savings alone could make the process economically viable. Other benefits include improved burst strength and tear indices of the product and reduced pitch deposition during the production process. The technology has focused on the white rot fungi, which have complex extracellular ligninolytic enzyme systems that can selectively remove or alter lignin and allow cellulose fibers to be obtained. Although still far from completely understood, these enzyme systems are being characterized mechanistically and on a molecular level with primary emphasis on the enzymes lignin peroxidase, manganese peroxidase and laccase. Scale-up to industrial process requirements presents challenges that are difficult to simulate in laboratory or pilot-scale tests. Inoculation, aeration and heat dissipation are key parameters for maintaining fungal activity. It may be possible to monitor and maintain consistent treatments through a program of active wood chip pile management. Overcoming these challenges will determine, in large part, if biopulping becomes a reality.

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Abbreviations

GLOX	glyoxal oxidase
LiP	lignin peroxidase
MnP	manganese peroxidase
VA	veratryl alcohol

Introduction

The objective of pulping is to extract cellulose fibers from plant material, generally hard or soft wood trees. Two approaches have been employed to pulp wood: mechanical pulping; and chemical pulping. Recent reports show that a biologically based approach has potential for improving both the economics and environmental impact of pulp generation [1]. Traditionally, cellulose removal has been accomplished by a mechanical process that has been improved over time by modifications such as refiner mechanical pulping and thermomechanical pulping. Although efficient, these methods are extremely energy intensive. Chemical pulping dissolves lignin from the cellulose and hemicellulose fibers. The primary chemical pulping process employed today is the kraft process, in which wood chips are cooked in a solution containing sodium hydroxide and sodium sulfide. The yield from chemical processes is generally lower than mechanical pulping due to some degradation of cellulose. A second drawback to chemical pulping is the large amounts of potentially hazardous chemicals which pose a danger to both mill workers and the environment [2].

Biopulping technology is based on the natural turnover of plant organic material. The process begins with fungal colonization of the xylem of exposed wood. Microscopic studies reveal penetration and degradation of the lumen, and softening and swelling of cell walls [3••]. Expansion of fungal hyphae and colonization through the parenchyma allow the organism to become established. As fungal degradation progresses the middle lamella separates from the cellulose-rich secondary wall structure.

Data suggest that biopulping has the potential to be an environmentally and economically feasible alternative to current pulping methods [3••,4]. Laboratory studies with *Ceriporiopsis subvermispora* on aspen and loblolly pine showed energy savings of 47% and 37%, respectively [5]. A decrease in extractable triglycerides, which are responsible for sticky deposits during paper production, was also documented [5]. The only undesirable property noted was a decrease in brightness of biologically-treated pulps.

Due to its high molecular weight, structural complexity and relative insolubility, lignin is a recalcitrant compound. Its degradation in wood appears to be limited to select fungi (bacterial degradation of non-wood lignins has been reported) [6]. White-rot basidiomycetes, however, are capable of degrading all wood components. Their ability to preferentially degrade a higher proportion of lignin than cellulose has resulted in intensive studies to optimize the catabolic potential of these organisms for biological pulping application. Despite extensive study, lignin degradation itself is still a poorly understood process. Recent advances in the biology of the white-rot fungi have shed new light on the process of biological pulping. This review summarizes these advances as well as the obstacles that remain as significant barriers to the widespread use of this technology.

Lignin degradation

Lignin is an extremely complex, three dimensional heteropolymer made up primarily of phenyl propane units, which serves as a structural component of higher plants. Lignin may account for up to 25% of the dry weight of woody plants. Lignin is randomly synthesized from coniferyl, p-coumaryl and sinapyl alcohol precursors [7]. As stated by Kirk, "The [enzyme] system must differ fundamentally from those involved in the biodegradation of all other important biopolymers, and meaningful analogies can not be readily drawn with other systems" [6]. Due to the large size of the polymer, degradation must take place in an extracellular fashion. Lignin is not degraded under

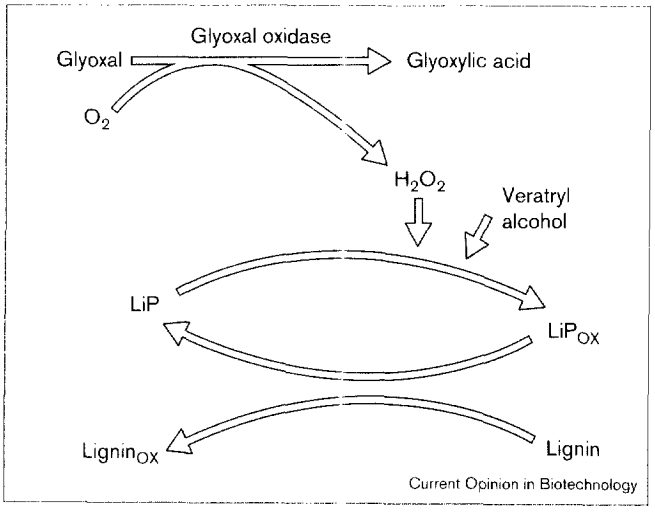
anaerobic conditions; the carbon–carbon and ether bonds joining subunits together must be cleaved via an oxidative mechanism. Complex but non-specific enzyme systems are needed for lignin to be degraded.

White-rot basidiomycetes tend to harbor gene families encoding the key enzymes responsible for depolymerization of lignin: manganese peroxidase, lignin peroxidase (LiP) and laccase [8]. The existence of gene families for these extracellular enzymes may be a consequence of their diverse roles in fungal physiology. Laccases, for example, have functions in plant pathogenesis, sporulation, and pigment production [9]. The abundance of enzymes may also be a result of the non-uniformity of the substrate they oxidize. In addition to these key enzymes, veratryl alcohol (VA), a degradation product of lignin, and glyoxal oxidase (GLOX), an extracellular peroxide-generating enzyme, also have important functions in lignin degradation. The primary enzymes involved in lignin degradation are discussed below.

Lignin peroxidase

The LiP system from *Phanerochaete chrysosporium* has been the most thoroughly studied and, unless otherwise stated, information presented herein was gathered with this organism. LiP (EC1.11.1.7) is a heme-containing glycoprotein secreted during secondary metabolism as a response to nitrogen limitation. LiPs are strong oxidizers capable of catalyzing the oxidation of phenols and aromatic amines, aromatic ethers and polycyclic aromatic hydrocarbons. LiP is initially oxidized by H_2O_2 to form a two-electron deficient intermediate termed compound I (Figure 1).Compound I oxidizes substrates by one electron and forms compound II, a more reduced enzyme intermediate. Compound II can in turn oxidize substrates by one electron and return the enzyme to its resting state [10].

Figure 1



Mechanism of lignin peroxidase action. OX, oxidized state.

LiP is too large a molecule to enter the plant cell wall. It appears that to accomplish degradation directly, LiP must attack exposed regions of the lumen. There is some uncertainty about this, however, as some microscopic studies indicate degradation of lignin occurs from inside the cell wall. The possibility of an indirect oxidation, where low molecular weight diffusable compounds, capable of penetrating the cell wall, are acted on by LiP and in turn oxidize lignin, has been proposed but is currently lacking evidence [11••].

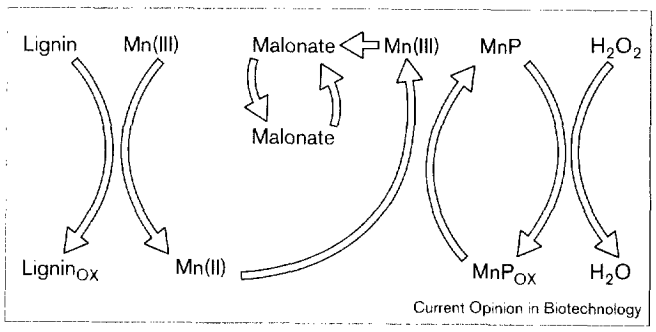
The theorized importance of LiP in delignification has decreased due to a number of observations summarized in detail by Archibald *et al.* [12]. Interestingly, LiP is not found in the most extensively studied biopulping fungus *C. subvermispora*, although *lip*-like gene sequences have been detected, and some have reported polymerization of lignin preparations rather than depolymerization when testing LiP *in vitro* [13].

Ten structurally similar LiP genes (designated *lipA* through *lipJ*) in three linkage groups have been identified in *P. chrysosporium* [14••]. The gene sequences are conserved among *lip* genes and the crystal structure of LiP proteins has been shown to resemble cytochrome C oxidase. Further complicating the studies of LiP regulation in *P. chrysosporium* is variation of gene expression from strain to strain [11••].

Manganese peroxidase

Manganese peroxidase (MnP; EC1.11.1.7) occurs in most white-rot fungi. Similar to LiPs, MnPs are glycosylated, heme-containing enzymes that functionally require H_2O_2. The first report on MnP was in *P. chrysosporium*. To date, five isozymes have been detected in *P. chrysosporium* MP-1 [11••]. The MnP system generates low molecular weight diffusable oxidizing agents capable of exerting their effect over a physical distance from the enzyme (Figure 2). It has also been reported that when Mn(II) is present, MnP will peroxidate unsaturated lipids causing the formation of lipoxyradical intermediates capable of oxidizing non-phenolic model

Figure 2



Mechanism of MnP action. MnP oxidizes Mn(II) to Mn(III). Mn(III) will then react with bidentate organic acid chelators such as malonate to form a complex capable of oxidizing phenolic residues in lignin [11••,12]. OX, oxidized state.

lignin compounds [11••]. Jensen *et al.* [15] suggested that MnP lipid-mediated peroxidation is responsible for the production of benzylic fragments from β-*O*-4 lignin structures by *C. subvermispora* when no LiP activity was detected. *C. subvermispora* appears to produce as many as 11 isozymes of MnP.

DNA sequence studies and protein crystallographic studies show that MnPs have a great deal of similarity to LiPs. As with LiPs, it has been difficult to get heterologous expression of MnPs in alternative hosts. DNA sequence analyses indicate that *mnp* genes contain both metal response and heat-shock response elements. In *P. chrysosporium*, MnP can be differentially regulated by carbon and nitrogen levels. Additionally, Mn(II) can induce transcription of *mnp1* and *mnp2*. Conversely, *mnp3* does not appear to be regulated by Mn. Godfrey *et al.* [16] constructed a reporter gene system for monitoring MnP gene expression. Their study demonstrated the anticipated responses from an *mnp* promoter and should have utility for determining conditions for optimal *mnp* gene expression from certain loci.

Veratryl alcohol and glyoxal oxidase

VA and GLOX are components of the peroxidase enzyme systems. VA is a secondary product of fungal metabolism that has been reported to stimulate lignin degradation. Its mode of action was at first hypothesized to be similar to the mediator compounds of the laccase system. Mediators are low molecular weight compounds oxidized by ligninolytic enzymes, which can then diffuse in to the plant cell wall to accomplish lignin oxidation. It is now believed, however, that the role of VA is to recycle the LiP and prevent its inactivation by H_2O_2 [17]. As stated above, LiP-compound I is a two electron intermediate that forms compound II when it uses one electron to oxidize substrates. Compound II will then oxidize substrates by one electron; however, in the presence of poor substrates and excess H_2O_2, compound II is converted to compound III, an inactive form of LiP. VA is a favorable substrate for compound II and functions to convert it back to its original state, thus recycling LiP and insulating it from inactivation by H_2O_2. The hypothesis that VA could serve as a diffusable oxidizer was not valid because of the instability of the VA cation radical. Cancel *et al.* [18] showed that VA-enhanced LiP activity is not due to *de novo* LiP synthesis by monitoring mRNA transcription from LiP isozymes H2 and H8. This evidence is strongly supportive of a protective role for VA rather than an enhancement of LiP gene expression. Collins *et al.* [10] demonstrated that tryptophan can function in the same fashion as VA.

H_2O_2 required for catalysis by peroxidases is most probably generated by GLOX. GLOX is an extracellular enzyme that acts by transferring electrons from low molecular weight aldehydes to O_2, resulting in the formation of H_2O_2 [11••]. Substrates for GLOX include glyoxal and glycoaldehyde, extracellular metabolites secreted by *P. chrysosporium*. GLOX genes have been cloned from *P. chrysosporium*. Kersten *et al.* [19] were able to show expression and secretion of GLOX in *Aspergillus nidulans*, when under control of a maltose-inducible expression system, at levels 50-fold greater than in *P. chrysosporium*. Aryl alcohol oxidase is also an H_2O_2 generating enzyme. It catalyzes the conversion of benzyl alcohols to the aldehyde and in the process transfers electrons to O_2 generating H_2O_2 [11••].

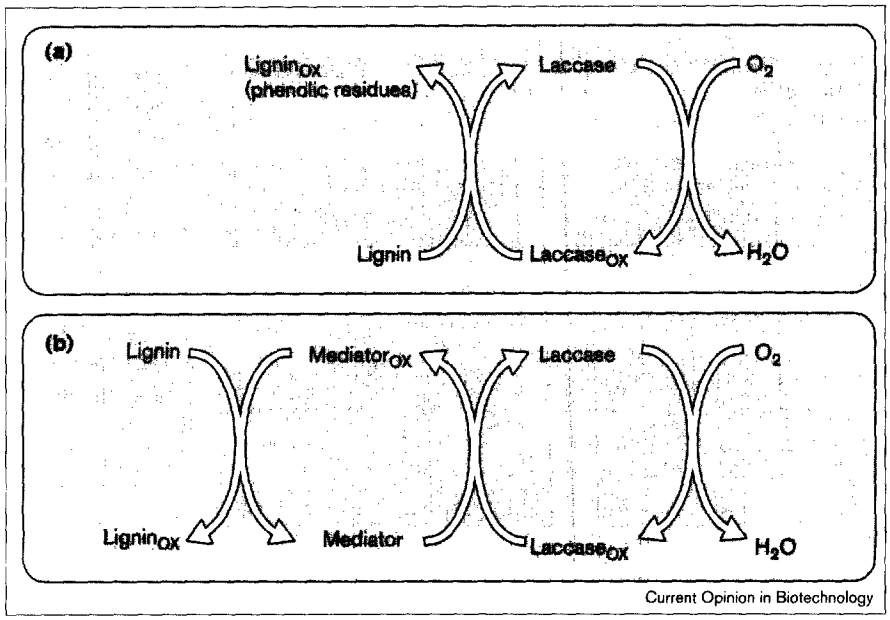
Laccase

A number of laccases have been found in a diverse variety of fungi, including not only wood-rotting basidiomycetes but well studied strains of non-ligninolytic ascomycetes from the genera *Aspergillus* and *Neurospora* [20]. Laccases have also been found in plants where they play a role in lignin synthesis. Unlike the peroxidases, heterologous gene expression does not appear to be problematic with laccases. Laccases harbor great biotechnological potential and alternative applications for laccases include pulp bleaching, effluent detoxification, washing powder components, removal of phenolics from wines and transformation of antibiotics and steroids [21]. *P. chrysosporium*, perhaps the most proficient producer of extracellular ligninolytic enzymes, was, until recently, not believed to produce laccase. Laccase activity has been detected but it is relatively low and repressed by glucose [13]. Laccases (benzenediol:oxygen oxidoreductases, EC1.10.3.2) are multi-copper-containing enzymes that oxidize phenolic compounds. Laccase reduces molecular oxygen to water accompanied by a one electron oxidation of an aromatic substrate (Figure 3). For example, several phenothiazines capable of forming cation radicals have been tested; however, to date, molecules in the class appear too unstable to act as effective mediators.

Laccase may interact directly with phenolic components of lignin or, in the presence of a 'mediator' compound, react with a broader range of substrates. A mediator is a co-substrate, the most often cited is 2,2′-azinobis-(3)-ethylbenzythiazoline-6-sulphonate (ABTS), which functions as a diffusable lignin-oxidizing agent. The existence of mediators was postulated because of the fact that purified laccases can not react directly with the intact fiberous cell wall. This indicated that another component was necessary and mediators were hypothesized to be low molecular weight 'oxidizing vehicles' secreted by fungi [22,23]. A natural mediator, 3-hydroxyanthranilic acid, has been detected in the white-rot fungus *Pycnoporus cinnabarinus* but little more is known about laccase mediators in nature [13]. The paucity of information on the natural occurrence of laccase mediators has not slowed the development of effective laccase-mediator systems to enhance ligninolytic activity [12,23,24]. For example, several phenothiazines capable of forming cation radicals have been tested; however, to date, molecules in the class appear too unstable to act as effective mediators.

Figure 3

Mechanism of **(a)** laccase action, and **(b)** laccase action via a mediator. OX, oxidized state.



More than 17 laccase genes from a variety of white-rot fungi have been cloned and sequenced [11••]. Most organisms contain multiple laccase genes, at least five in *Trametes villosa* and four in *Rhizoctania solani*. Mansur *et al.* [9] recently showed that at least seven laccase isozymes occur in a recently characterized white-rot isolate, designated I-62. These gene families contain closely related proteins that differ slightly in their substrate spectrum or regulation. Laccase has been cloned and expressed in *Saccharomyces cerevisiae*. Carbon source can induce laccase activity and regulation of laccase expression can differ greatly among species [9]. Investigators have induced laccase activity by heat and cadmium exposure and suggested that laccase induction may be part of some stress responses [25]. The influence of nitrogen on laccase activity has varied from organism to organism and remains controversial.

Process applications

Biological pulping has been an area where it has been stated that there is a need for a 'breakthrough technology' [26]. To a large extent, the mechanism of biopulping is still relatively mysterious. The greatest biopulping effect, as assessed by reduction in energy consumed in subsequent refining, can be seen at a very early stage of the process. At this stage wood looks morphologically no different to untreated wood. This suggests that the structural components, including lignin, are still present after treatment yet much less energy is required to refine this material [5]. Although the potential for application of fungal treatment has been demonstrated, a number of engineering and process alterations must be incorporated to exploit this technology.

No single organism is ideal for all biopulping applications. *P. chrysosporium* appears to be effective for hardwood, whereas *C. subvermispora* holds potential for both hard and softwood. *Phlebiopsis gigantea* has been shown to rapidly colonize freshly cut logs from conifers and inhibit colonization by sapstain fungi (which cause discoloration hence lowering pulp quality) [27]. The organisms of choice must be capable of accomplishing delignification in a reasonable period of time, delignification must be selective (i.e. leaving the cellulose fibers intact) and the organisms must be robust enough to thrive in conditions that vary considerably within the biopulping device. Selectivity can be difficult to maintain. Messner and Srebotnik [5] cited a report describing extensive selective delignification of whole logs (the logs were then used for cattle feed) but this appears to be a highly unusual case. Fungi selected for rapid delignification appear to be the best candidates for biopulping as they have a tendency to demonstrate selective delignification (i.e. leaving cellulose fibers untouched), as exemplified by *C. subvermispora*.

It is known that significant changes in the cell-wall structure take place at a very early stage in the biopulping process. Cells retain their morphology but do not react with lignin stains (which are used to assess delignification microscopically). Laboratory tests show the gradual penetration of proteins of increasing molecular mass into the cell as the biopulping process progresses; however, the cells are not sufficiently permeabilized to allow penetration of ligninolytic enzymes [11••]. As previously mentioned, evidence indicates that diffusable lignin-oxidizing intermediates are generated. The frequency and regulation of this process relative to 'simultaneous decay' (degradation of cellulose, hemicellulose and lignin) is unknown. Considerable attention is now being directed toward regulation of gene

expression in ligninolytic fungi and should yield information on optimizing selective decay.

Selective delignification (which can be assessed microscopically using differential staining [28]), can be highly variable as fungi can remove lignin at one site on a wood chip while removing lignin and cellulose at another site on the same chip. Fungi, such as *P. chrysosporium*, will switch back and forth from selective to non-selective degradation, possibly as a function of environmental conditions. Efforts to employ cellulase-defective mutants as means to attain selectivity have not been very successful [3••].

The indigenous microbial populations harbored on wood chips present most white-rot fungi with a competitive challenge. *P. chrysosporium* appears to be one of the more resilient of the white-rot fungi in contrast to *C. subvermispora*, which can not become established in the presence of indigenous microorganisms. Because of this problem, most regimes for biological pulping incorporate a decontamination step: both heat and chemical treatments have been shown to be effective [1,3••,5,29••]. Application of steam for ten minutes gave results comparable to autoclaving, and steaming for as little as 15 seconds was sufficient to allow the establishment of a *C. subvermispora* inoculum [3••]. Successful inoculation and colonization can be promoted by the introduction of additional nutrients, for example, corn steep liquor has been shown to enhance biopulping and reduce the size of inoculum needed from 3 kg per ton to less than 5 g per ton of wood chips [29••].

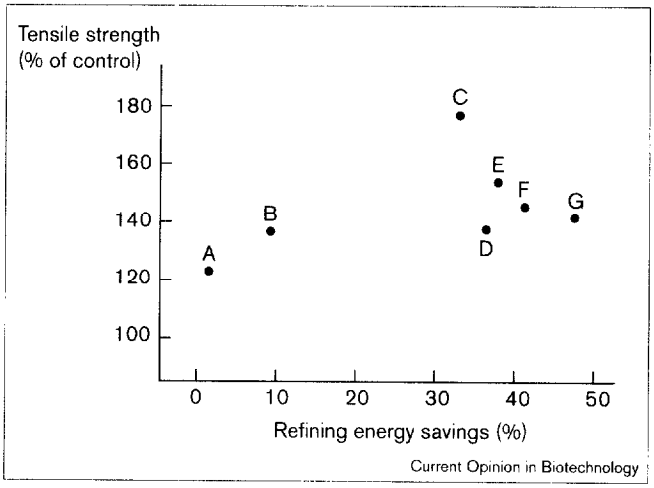
Whether fungal colonization and growth are successful depends on the prevailing environmental conditions in the wood chips. Among important parameters are aeration and moisture content of the chips. Heat, generated by fungal metabolism, can adversely affect the process. The optimal temperature for *C. subvermispora* is 27–32°C; however, the temperature of the interior of wood piles can reach 42°C in six days [30••]. Thus, a successful biopulping process will include a mechanism for dissipating excess heat in order to maintain fungal activity [3••].

The problems summarized above become even more daunting when the transition from pilot-scale to full-scale operation is taken into account. Pulp mills process 200–2,000 tons of wood each day and, as a result, there can be wide variation among the piles of chips due to heat, aeration, or contamination by other microorganisms. These and other factors (e.g. chip compression in the pile and moisture content) can have a direct impact on the quality of the product generated by the biopulping process. Scott *et al.* [29••] suggested that these problems may be overcome with minor modifications to equipment already on site at most pulp mills. Screw conveyers already in use for chip transport can be adapted to cool and inoculate the chips. Ventilation and uniformity of chip treatment go hand-in-hand in assuring a consistent product.

Economic considerations

More than a 25% saving in energy costs should be expected from refiner operation following fungal treatment. Operating costs and energy savings were calculated for a 200 ton per day mill by Scott *et al.* [29••]. A two-week incubation could not only reduce energy consumption, but improve the economic picture in other ways. Pulps can be blended to form a furnish (specific mix of raw materials, such as pulp and chemicals, to make a particular grade of paper) that will increase the strength of the finished product. Kraft pulp is often blended with thermomechanically produced pulp to improve strength qualities. The improved strength properties of biopulped material could reduce the amount of more expensive kraft pulp needed to produce the desired furnish and further reduce production costs [30••]. Figure 4 shows the effects of various ligninolytic fungal treatments on energy saving and tensile strength.

Figure 4



Relationship between outcomes of treating pulps with selected fungal species based on tensile strength and energy savings. A, *Trametes versicolor*; B, *Dichomitus squalens*; C, *Phlebia tremellosa*; D, *Pholiota mutabilis*; E, *Phanerochaete chrysosporium*; F, *Phlebia subserialis*; G, *Phlebia brevispora*. Adapted from [31].

Fungal pretreatment results in a significant reduction in the pitch content of the wood chips [29••]. Pitch is problematic in paper making where it can cause insoluble deposits on the paper-making system. Pitch is problematic in both chemical and mechanical pulping, and process chemicals (also referred to as 'dispersants') must be used to control it. When uncontrolled, pitch can be responsible for breaks in the paper roll and a decrease in paper strength leading to machine shut downs. The economic benefits derived from pitch reduction have not as yet been quantified.

Although most studies have tested the effect of fungal treatment on mechanical pulping, chemical pulping could also be enhanced by the process. Evidence shows that both the kraft and sulfite pulping processes could be augmented by fungal treatments of wood chips [5]. Improved penetration

of the cooking compounds due to fungal treatment may be responsible for the increased efficiency. The benefits derived include reductions in the amount of chemicals required for processing and reduced cooking time resulting in a decrease in the amount of bleaching chemicals needed in the next production step. Studies on the effect of a biotreatment prior to kraft pulping have shown a reduction in Kappa number (i.e. lignin content) and increase in tensile strength; however, brightness was decreased. Messner and Srebotnik [5] tested five ligninolytic fungi for their effect on sulfite pulping. They showed that a two-week treatment with *C. subvermispora* dramatically decreased the Kappa number after sulfite cooking. Studies indicate no delignification or alteration of cell walls occurred. This suggested that lignin was modified in such a way as to make it more susceptible to sulfite cooking.

Conclusions

Certainly, some engineering challenges are still daunting. Process parameters, such as asepsis, aeration and nutrient addition, will depend, to a large extent, on data generated by studies on selective decay. These data will, in turn, show the ultimate economic and environmental effects of fungal treatments on the process. It does not appear that major reengineering or capital investment would be required. Time constraints are often cited as a drawback to biopulping. The fact that significant alterations of wood structure occurs within two weeks indicates that there are reasonable ways to overcome this drawback. For example, wood is routinely aged or 'seasoned' to minimize pitch — biopulping could be integrated into this process.

Biopulping has the potential to be an environmentally benign means of improving both the economics of pulp production and the quality of pulp produced. Fungal pretreatment can produce significant energy savings for producers of mechanically manufactured pulp and decrease chemical consumption in chemical pulping operations.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Scott GM, Akhtar M, Swaney RE: **Economic evaluation of biopulping.** In *Biotechnology in the Pulp and Paper Industry: 7th International Conference: 1998 June 16–19; Vancouver.* Vancouver: 1998:B3-6.

2. Smook GA: *Handbook for Paper and Pulp Technologists.* Atlanta: TAPPI;1989.

3. •• Akhtar M, Blanchette RA, Myers G, Kirk TK: **An overview of biomechanical pulping research.** In *Environmentally Friendly Technologies for the Pulp and Paper Industry.* Edited by Young RA, Akhtar M. New York: John Wiley & Sons; 1998:309-340.
A state-of-the-art review of the technological advances made towards bringing biological pulping to large-scale operation. The review includes comparisons of paper property changes as a result of biological pulping and issues such as chip pile aeration and asepsis.

4. Akhtar M, Scott GM, Lentz MJ, Horn E, Swaney RE, Kirk TK: **Commercialization of biopulping for mechanical pulping.** In *Biotechnology in the Pulp and Paper Industry: 7th International Conference: 1998 June 16–19; Vancouver.* Vancouver: 1998: AA55-58.

5. Messner K, Srebotnik E: **Biopulping: an overview of developments in an environmentally safe paper-making technology.** *FEMS Microbiol Rev* 1994, **13**:351-364.

6. Kirk TK: **Degradation of lignin.** In *Microbial Degradation of Organic Compounds.* Edited by Gibson DT. New York: Marcel Dekker; 1984:399-437.

7. Tuor U, Winterhalter K, Fiechter A: **Enzymes of white-rot fungi involved in lignin degradation and ecological determinants for wood decay.** *J Biotechnol* 1995, **41**:1-17.

8. Yaver DS, Feng X, Golightly EJ, Brown KM, Brown SH, Rey MW, Schneider P, Halkier T, Mondorf K, Dalboge H: **Purification, characterization, molecular cloning and expression of two laccase genes from the white rot basidiomycete *Trametes villosa*.** *Appl Environ Microbiol* 1996, **62**:834-841.

9. Mansur M, Suarez T, Fernandez-Larrea JB, Brizuela MA, Gonzalez AE: **Identification of a laccase gene family in the new lignin-degrading basidiomycete CECT 20197.** *Appl Environ Microbiol* 1997, **63**:2637-2646.

10. Collins PJ, Field JA, Teunissen P, Dobson AD: **Stabilization of lignin peroxidases in white rot fungi by tryptophan.** *Appl Environ Microbiol* 1997, **63**:2543-2548.

11. •• Kirk TK, Cullen D: **Enzymology and molecular genetics of wood degradation by white rot fungi.** In *Environmentally Friendly Technologies for the Pulp and Paper Industry.* Edited by Young RA, Akhtar M. New York: John Wiley & Sons; 1998:273-308.
This review discusses the components of wood as they are found in the raw substrate, including molecular structures and properties. A detailed summary of the biochemistry and genetics of ligninolytic systems and peroxide-generating enzymes is well rewritten and timely.

12. Archibald FS, Bourbonnais R, Jurasek L, Paice MG, Reid ID: **Kraft pulp bleaching and delignification by *Trametes versicolor*.** *J Biotechnol* 1997, **53**:215-236.

13. Eggert C, Temp U, Eriksson K-EL: **Laccase-producing white rot-fungus lacking lignin peroxidase and manganese peroxidase.** In *Enzymes for Pulp and Paper Processing.* Edited by Jefferies TW, Viikari L. Washington DC: American Chemical Society; 1996:130-150.

14. •• Janse BJH, Gaskell J, Akhtar M, Cullen D: **Expression of *Phanerochaete chrysosporium* genes encoding lignin peroxidases, manganese peroxidases and glyoxal oxidase in wood.** *Appl Environ Microbiol* 1998, **64**:3536-3538.
Expression of genes relevant to biopulping was quantified using magnetic beads to capture mRNA encoding ligninolytic enzymes. Data suggest that some genes previously believed to be highly expressed during biological pulping may be highly dependent on the species of wood being processed. For example, carbon starvation induces *lipD*, whereas nitrogen limitation appears to induce *lipA*, *lipC*, and *lipJ*. Unlike many studies, this investigation examined gene expression in wood rather than under cultural conditions. Data suggest that genes such as *lipD* and *LipE* are not actively involved in biopulping.

15. Jensen KA, Bao W, Kawai S, Srebotnik E, Hammel KE: **Manganese dependent cleavage of nonphenolic lignin structures by *Ceriopsis subvermispora* in the absence of lignin peroxidase.** *Appl Environ Microbiol* 1996, **62**:3679-3686.

16. Godfrey BJ, Akileswaran L, Gold MH: **A reporter gene construct for studying the regulation of manganese peroxidase gene expression.** *Appl Environ Microbiol* 1994, **60**:1353-1358.

17. Koduri RS, Tien M: **Kinetic analysis of lignin peroxidase: explanation for the mediation phenomenon by veratryl alcohol.** *Biochemistry* 1994, **33**:4225-4230.

18. Cancel AM, Orth AB, Tien M: **Lignin and veratryl alcohol are not inducers of the ligninolytic system of *Phanerochaete chrysosporium*.** *Appl Environ Microbiol* 1993, **59**:2909-2913.

19. Kersten PJ, Witek C, Vanden Wymelemberg A, Cullen D: ***Phanerochaete chrysosporium* glyoxal oxidase is encoded by two allelic variants: structure, genomic organization, and heterologous expression of *glx1* and *glx2*.** *J Bacteriol* 1995, **177**:6106-6110.

20. Bollag J-M, Leonwicz: **Comparative studies of extracellular fungal laccases.** *Appl Environ Microbiol* 1984, **48**:849-854.

21. Luisa M, Goncalves FC, Steiner W: **Purification and characterization of laccase from a newly isolated wood-decaying fungus.** In *Enzymes for Pulp and Paper Processing.* Edited by Jefferies TW, Viikari L. Washington DC: American Chemical Society; 1996:260-266.

22. Camarero S, Munoz C, Bocchini P, Escudero E, Martinez MJ, Martinez AT: **Enzyme/mediator systems based on *Pleurotus* laccase and MnP acting on wheat straw lignin after different substrate pretreatments.** In *Biotechnology in the Pulp and Paper Industry: 7th International Conference: 1998 June 16–19; Vancouver.* Vancouver: 1998:B183-B186.

23. Bourbonnais R, Paice MG, Freiermuth B, Bodie E, Borneman S: **Reactivities of various mediators and laccases with kraft pulp and lignin model compounds.** *Appl Environ Microbiol* 1997, **63**:4627-4632.

24. Srebotnik E, Jensen KA, Hammel KE: **Cleavage of nonphenolic lignin structures by laccase in the presence of 1-hydroxbenzotriazole.** In *Biotechnology in the Pulp and Paper Industry: 7th International Conference: 1998 June 16–19; Vancouver.* Vancouver: 1998:B195-B197.

25. Fink-Boots M, Wilkolazka A, Malarczyk E, Leonowicz A: **Changes in sets of laccase isoforms under stress conditions in *Cerrena unicolor*.** In *Biotechnology in the Pulp and Paper Industry: 7th International Conference: 1998 June 16–19; Vancouver.* Vancouver: 1998:B179-B1181.

26. Barker RG, Wann SR, Kaphammer BJ: **Biological sciences in the pulp and paper industry – the industry's viewpoint.** In *TAPPI Proceedings of the Biological Sciences Symposium: 1997; San Francisco.* Atlanta: TAPPI; 1997:1-6.

27. Behrendt CJ, Blanchette RA: **Biological processing of pine logs for pulp and paper production with *Phlebiopsis gigantea*.** *Appl Environ Microbiol* 1997, **63**:1995-2000.

28. Srebotnik E, Messner K: **A simple method that uses differential staining and light microscopy to assess the selectivity of wood delignification by white rot fungi.** *Appl Environ Microbiol* 1994, **60**:1383-1386.

29. •• Scott GM, Akhtar M, Lentz MJ, Swaney RE: **Engineering, scale-up, and economic aspects of fungal pretreatment of wood chips.** In *Environmentally Friendly Technologies for the Pulp and Paper Industry.* Edited by Young RA, Akhtar M. New York: John Wiley & Sons; 1998:341-383.

A review article discussing the engineering and scale-up aspects currently hampering the implementation of this technology. A cost analysis is included which considers time, production bottle necks and energy savings.

30. •• Scott GM, Swaney R: **New technology for paper making: biopulping economics.** *TAPPI J* 1998, **81**:153-157.

A schematic diagram and cost analysis of a biopulping process for a 600 ton per day mill is presented. The value of reduced kraft pulp usage and increased throughput are taken into consideration.

31. Reid ID: **Determining molecular weight distribution of lignins and their biodegradation products by gel filtration on a high-performance agarose column with a mixed ethanol-aqueous alkali solvent.** *Trends Biotechnol* 1991, **9**:262-265.