Chapter 12

Enzyme Processes for Pulp and Paper: A Review of Recent Developments

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The pulp and paper industry is applying new, ecologically sound technology in its manufacturing processes. Many interesting enzymatic applications have been proposed in the literature. Implemented technologies tend to change the existing industrial process as little as possible. Commercial .applications include xylanases in prebleaching kraft pulps and various enzymes in recycling paper. In the future, value-added products could be built around enzyme processes. When new applications are proposed that do not fit into existing practices, either the process or the enzyme must be altered. The decision depends on process economics and the feasibility of changing the technology. We review here new applications of enzymes in the pulp and paper industry and how they might be changed to implement the technology on an industrial scale. Also, this review suggests how existing enzymes may be used by process engineers to improve the efficiency of unit operations, the pulp products, or both.

This is a difficult time for the pulp and paper industry. Consumer standards are high, and manufacturing is competitive. Pulp production is

increasingly derived from tropical regions (*1*). Cost reduction pressures are causing consolidation of companies through mergers and acquisitions; many research and development laboratories are being downsized, closed, or directed toward short-term objectives and opportunities; and profitability is being constrained by external factors including globalization, environmental concerns, and competition (*2*). Pulp and paper is a large industry that is highly capital-intensive and has been periodically affected by overcapacity (*1*). In the United States, more than 300 kg of paper are consumed annually per capita (*3*). To maintain this level of supply, we need to find new ways to use our forest resources more efficiently and with fewer environmental consequences. Emerging technologies based on sustainable use of renewable resources hold promise for the rejuvenation and growth of the pulp and paper industry.

Enzyme Applications in Pulp and Paper

Biotechnology has the potential to increase the quality and supply of feedstocks for pulp and paper, reduce manufacturing costs, and create novel high-value products. Novel enzyme technologies can reduce environmental problems and alter fiber properties. Because the pulp and paper industry is capital-intensive with facilities specific to the tasks (*1*), new technology must either reduce expenses or fit easily into the existing process design. Pulp and paper companies are reluctant to build or expand plants when the overall industry has enough capacity to satisfy market demands (*1*). Nevertheless, the industry has embraced enzymes for use in the paper-making process.

Environmental and Manufacturing Benefits

While the size and commodity nature of many of its products make changes difficult, the pulp and paper industry has responded to environmental and economic concerns. It implements new technology as required and when economics dictate change (*4*). For example, in response to environmental concerns and regulations, the industry has greatly reduced chlorinated aromatic byproducts that can be formed during pulp bleaching (*5-7*), first by reducing the amount of residual lignin in pulps and second by turning to other bleaching agents (*8*). An enzyme technology based on microbial xylanases has helped to achieve this goal by reducing or even eliminating the need for chlorine in the manufacture of elemental chlorine free (ECF) and totally chlorine free (TCF) printing and writing paper grades (*8-22*). Enzymes have helped meet environmental goals in other ways as well. By reducing costs involved in

deinking (*12*), enzymes have increased the ability of manufacturers to recycle fiber, thereby placing fewer demands on timber resources. Enzymes have been used commercially to reduce paper manufacturing costs or improve the product. Lipases can control the accumulation of pitch during the production of paper from pulps with high resin content, such as sulfite and mechanical pulps from pine (*13*). Enzymes also help remove contaminants in the recycle stream, They can reduce the accumulation of adhesives and pitch residues, called stickies, on machines (*14*). They can facilitate the deinking of recycled paper and improve pulp drainage, which is particularly important as the amount of recycled fiber in the feedstock stream increases. With higher drainage rates, paper machines are able to operate faster, which again saves capital costs (*5,13,15,16*).

Xylanases have saved chemical costs for the industry (*7*) without interfering with the existing process. This technology has increased the bleaching speed in both TCF and ECF processes (*17*) and, in the case of chlorine dioxide bleaching, has actually increased the throughput of the plant due to debottlenecking at the chlorine dioxide generator (*7*). Developments of this last type are viewed very favorably since they enable the industry to make better use of its existing capital equipment.

Innovation and Implementation

Many other enzyme applications are possible based on properties demonstrated in the laboratory. These include eliminating caustic chemicals for cleaning paper machines, enhancing kraft pulping, reducing refining time, decreasing vessel picking, facilitating retting, selectively removing fiber components, modifying fiber properties, increasing fiber flexibility, and covalently linking side chains or functional groups. Commercial development of these applications will require better knowledge of enzyme mechanisms and actions on fibers, development of improved processes for their use, and changing enzymes to function better under existing operating conditions.

Efficacy is critical. The effect must be sufficient and reliable enough to warrant a change in process technology, or it will not be used. Economy of scale can greatly reduce the expense of enzyme production but unless the benefit is substantial, a paper maker will not implement the technology. Technical barriers can also block implementation. The use of an enzyme in a process step might call for adjusting operating parameters such as pH, adding chemicals such as surfactants, or making capital expenditures such as mixing tanks. In addition to the cost to implement the enzyme technology, the changes required for altered downstream processing might create expenses. For these reasons, innovative enzyme processes are most readily accepted when they can be incorporated into existing practices.

Often the biological or biochemical basis for an enzyme application is not well understood and must be developed through an empirical approach. For example, the ambient pH and temperature of kraft pulps dictate that xylanases should exhibit both alkaline activity and thermostability, but other factors make similar enzymes better suited to enhance bleachability. Xylanase use must be tested and levels of enzyme, the source of the enzyme, and location of enzyme treatment in the process are all critical factors to the successful deployment of the technology at the mill (*18*). The particular pulp or fiber might require the use of specific enzymes or doses. The pH optimum, the temperature of operation, and the sensitivity of the enzymes to other components of the process might all have to be modified.

Twenty years ago, the only means to make these changes would be to find new organisms and new enzymes. Today, these traditional means are available, and in addition, recombinant DNA has allowed the cloning of enzymes from known producers, difficult-to-culture microorganisms, and even unculturable organisms. These cloned enzymes can be modified for temperature, pH, and stability using similar techniques. Random mutagenesis, gene shuffling, directed evolution, and site-specific changes in the active site or supporting structure have all allowed the biotechnologist to change the natural enzyme to fit the need. The advent of *in silico* design adds another dimension to the possibilities by allowing the testing and design of enzymes that are not presently found in nature.

Pulp and Paper Processes

This review will present selected applications of enzymes in pulp and paper processing. Pulping starts with the conversion of wood or agricultural materials into a flexible fiber that can be made into paper. Depending on the ultimate application, many pulping processes can be used. The most common, kraft pulping, is a hot alkaline sulfide digestion of wood that removes most of the lignin and leaves a cellulosic fiber that is flexible yet strong. Acid sulfite pulping is also used to produce printing and writing paper grades. These chemical pulping processes form the basis for the production of printing, writing, and packaging paper grades. Mechanical, thennomechanical, and chemo-thermomechanical pulping produces fibers in much higher yield but with lower strength and optical properties. Such fibers can be incorporated into newsprint or magazine stock. After pulping, the fiber is washed and then bleached if the end use calls for white paper. Many other manipulations such as sizing, addition of fillers, and color addition can take place after this to produce the final paper product.

In addition to primary fiber, recycled fiber derived from recovered postconsumer paper is a major fiber source. Basic steps in paper recycling are pulping, washing, screening and flotation (to remove ink and other contaminants), bleaching, and wastewater cleanup. Enzymes can be useful in each of these process steps. The remainder of this review will describe enzyme uses in these processes and will also touch on the alteration of enzymes to better fit the process needs.

Enzyme Use in Pulping

Enzymes used in pulping can increase the yield of fiber, lessen further refining energy requirements, or provide specific modifications to the fiber. Cellulases have been used in many processes in the paper industry. Enzyme pretreatments using cellulase, hemicellulase, and pectinase have been shown to enhance the kraft pulping of sycamore chips and other pulp sources (*19-21*). This enzyme mixture allowed for better delignification of the pulp and savings in bleaching chemicals without altering the strength of the paper. The cost of the enzymes and questions about the effectiveness of a large enzyme aiding the low molecular weight pulping chemicals (*16*) has led to skepticism about the implementation of cellulases to enhance the kraft process.

Cellulases have been tested on mechanical pulps as well. In this case, there are conflicting results shown by a benefit in brightness and an increase in energy required for refining (*22*) using a crude cellulase on radiata pine compared with reduced energy required after a cellobiohydrolase treatment on spruce mechanical pulps (*23,24*). Other enzymes such as laccase and protease have been reported to reduce energy requirements in mechanical pulping (*16*). Laccase treatment has the additional benefit of increasing fiber bonding, which enhances the strength of the paper (*25*).

The use of enzymes in the refining of virgin fibers has been ongoing for decades. Kraft pulp has been treated with cellulases and xylanases, and both enzymes have reduced the energy required for further refining (*26*). The cellulases must be used carefully so they do not reduce the strength of the fibers (*27*). Xylanase treatments are more effective on unbleached pulps than they are on bleached kraft pulps (*16*).

The yield of thermomechanical pulp can also be increased by the use of enzymes. De-esterifying the soluble O-acetyl-galactoglucomannans of Norway spruce using an acetyl esterase was shown to precipitate the galactoglucomannans onto the fiber and increase the yield of fiber from the process (*28*).

Dissolving pulps are derived from pulps that contain a high level of cellulose. These pulps are treated to form soluble reactive carbohydrate chains that are then extruded into fibers or films. Endoglucanase treatment decreased the viscosity and chain length and increased the reactivity of a pulp made from eucalyptus and acacia (*29*). The endoglucanases were more efficient at hydrolyzing the pulp than were cellobiohydrolases at the same protein dosage (29). The use of cellulases in a two step process, where the alkali insoluble material was recovered, treated with enzymes, and recombined with the first extraction, resulted in a dissolving pulp that was more soluble in alkali than pulp treated directly with enzymes followed by alkali. Although when compared at the same level of hydrolysis, the fibers from the two stop process were actually slightly lower in solubility (*30*). Alkali extraction of dissolving pulps made from bleached hardwood kraft fiber and recycled paper rich in hardwood fiber, followed by xylanase treatment and a second alkali extraction, provided a reduction in the hemicellulose content of the pulp, acceptable viscosity, and alkali solubility (*31*).

Enzyme Use in Bleaching

Pulp bleaching has been repeatedly targeted as an application for enzymes in pulp and paper processing. The goal of bleaching is to whiten the pulp by changing or removing colored components. Initially, lignin-degrading enzymes were considered most important. In 1986, however, Viikari et al. (*10*) demonstrated that xylanases were effective as a prebleaching agent for pulps. In more recent years, laccases or manganese peroxidases, either alone or in combination with low molecular weight mediators, have been examined for their usefulness in pulp bleaching.

Xylanases in Prebleaching

The use of xylanases for prebleaching kraft pulp has been one of the greatest success stories of enzymes in the pulp and paper industry. Enzyme use helps to solve some of the environmental concerns associated with the use of chlorine in bleaching. The subject has been reviewed many times (*5,6,9,15,32*-35) and will not be covered here except for more recent developments. The mechanism by which xylanase assists in bleaching has come under study (*9,11*). Xylanase treatment can improve lignin extraction, alter carbohydrate and lignin associations, or cleave redeposited xylan (*9,36,37*).

One UV absorbing material, hexenuronic acid, is formed during kraft pulps from 4-methyl-glucuronic acid residues present on the xylan (*38-40*). The cleavage of the xylan allows the removal of such groups, thus saving on chemicals that would be needed to bleach those residues (*41*). An additional mild acid treatment will also selectively remove these colored components (*42*,*43*). The removal of the hexenuronic acid by xylanase treatments also helped to prevent brightness reversion of the treated kraft pulps (*44*).

Other studies on fiber modification have indicated that xylanase treatment allows alkali better access to the lignin (*45*). This enables more of the lignin to be removed and improves the bleaching efficiency for the remaining lignin in the fiber (*46*). The xylanase could be removing xylan that blocks access to the lignin, or xylan could precipitate on the surface of the fiber during kraft pulping, thereby preventing lignin extraction. This improved lignin removal and the removal of the bleach-consuming hexenuronic acid might explain the mechanism of xylanase prebleaching (*47*). However, there is evidence that xylan does not necessarily reprecipitate on the surface in all pulps (*48*), and our understanding of the xylanase bleaching mechanism at this point does not provide a means of predicting if bleaching will be enhanced by a given xylanase. Even the demonstration of carbohydrate (xylan) removal does not always correlate with bleaching efficacy (*49*).

Novel Xylanases

The potential applications of xylanases and the need for enzymes that might be more appropriate for use in pulp bleaching has caused many researchers to look for xylanases in different organisms and environments (*50*). Because the kraft process results in pulps that are alkaline and at higher temperatures, enzymes that do not require adjustment of temperature or pH are better suited to the process. Thermophilic (*50-55*) and alkaline sources (*56-58*) for these enzymes have been recently reviewed. Substantial amounts of work have been devoted to isolating and cloning new xylanases from all sources. Using xylanase as a search term in the scientific bibliographic databases, we found an average of 24 papers per year from 1982 to 1990. The same search from 1991 to 2000 revealed an average of 188 papers per year. Not all of these were papers about new xylanases, but a better indicator may be the number of xylanase-encoding DNA sequences that have been entered into data banks. A recent computer analysis (BLAST) of the nucleotide sequences with representatives of the Family 10 and 11 xylanases revealed more than 380 closely related protein-encoding sequences in the public databases.

Knowledge of the structure of a protein and its amino acid sequence might be used to explain why some xylanases have activity in alkaline conditions (59)

while others are active only in acidic conditions (*60*). Structural analysis of the *Bacillus circulans* xylanase provided insight into the unusually high pKa of an active site glutamate (*59*). The *Aspergillus niger* xylanase was active in acidic conditions due to the presence of an aspartic acid residue whereas other alkaline active xylanases have an asparagine residue (*60*). The knowledge gained from these studies can be used to better adapt xylanases to a given function. Alterations of xylanases to increase their activity under process conditions will be covered in a later section.

Variation in Applications

Xylanases have been used on a wide variety of pulp fibers. Pulps from softwoods (radiata pine, Douglas-fir, western hemlock, redcedar, loblolly pine, slash pine, black spruce) (*62-68*), hardwoods (aspen, sweetgum, oak, tupelo) (*65,66,69-72*), bagasse (*72,73*), eucalyptus (*67,74-80*), ramie fibers (*81*), bamboo (*82*), khar grass (*79*), and wheat straw (*83*) have all been treated with xylanases and the treatment appears useful in assisting the bleaching of the pulp.

Not all xylanases work with all pulp sources. Indeed, there have been several reports of some enzymes working better than others, and synergy among xylanases or other carbohydrases has also been shown (*84,85*). In general, most of the xylanases used for bleaching belong to the Family G (or 11) enzymes, which are smaller (*49,86-89*) than those in Family F (or 10). Some xylanases act better with other xylanases (*84,85*) or other carbohydrases (*90-94*). The method of pulp production may be important for the activity of other carbohydrases to improve pulp bleaching (*95*).

The source of the fiber is not the only variable in determining the use of xylanases in bleaching. One variable is the type of pulp being produced. Kraft, sulfite, dissolving, mechanical, and thennomechanical pulps are all possible. Xylanase treatment of kraft pulps is quite common and is well represented in the mentioned uses. Kraft pulping followed by oxygen delignification complicates the use of xylanase since the levels of enzymes must be empirically adjusted (*96*). Oxygen delignification makes more xylan accessible, and the enzyme doses for bleaching are actually lower than for kraft pulp.

Xylanase treatments have had mixed success with sulphite pulps. Repeated xylanase and alkaline oxygen treatments provided some increase in the ability to bleach sulphite pulp (*97*). The need for repeated enzymatic treatments without some method of enzyme recycle would make this economically unworkable. Xylanase treatment was tested on sulphite pulps bleached with hydrogen peroxide (98) and no benefit was shown. Reducing the residual pentose content of dissolving pulps was possible using xylanase, but success depended upon having a low level of pentosans present in the pulp and repeating treatment and

alkali extraction (*99*). Xylanase was able to enhance the brightness of a fungal-pretreated dissolving pulp (*100*).

There is also variability in the stage of the process where the enzyme treatment is performed. It could be pre- (*19*) or post-pulping, before or after alkaline treatment (*102*), or before or after the bleaching treatment. Alkaline leaching and enzyme treatment work by different, noncomplementary mechanisms, so it is not useful to combine the processes. The method of bleaching is also a variable, and improving bleaching with ozone, chlorates, peracetic acid, and peroxide is important for the industry (*102,103*). Use of enzyme treatments may have to be reoptimized when using a different bleaching method.

Other Enzymes Used for Pulp Bleaching

In general, the effect of xylanase on final pulp brightness is considered indirect. It removes reactants with the bleaching agent or obstacles to the bleaching action. A similar effect was shown for cellulase treatment and color removal in recycled yellow pages (*104*). There are some reports of direct brightening with xylanase treatments (*105, 106*), but most of the direct bleaching with enzymes is done using oxidative enzymes that directly attack the color-producing compounds in the lignin.

The enzymes that attack the lignin components of the fiber are oxidative. Peroxidases use hydrogen peroxide, and laccases use oxygen and eventually react with the lignin-derived moieties. The fungi *Trametes versicolor* and *Phanerochaete chrysosporium* degrade lignin in kraft pulp and enhance its brightness (*107*). *P. chrysosporium* produces manganese peroxidase (MnP) and lignin peroxidase (Lip). *T. versicolor* makes Lip, MnP, and laccase. Cellobiose dehydrogenase is also made by both fungi, and its role in wood degradation and potential applications has recently been reviewed (*108-112*).

Treating pulp with fungal enzymes partially enhanced brightness but did not brighten the pulp to the same extent as fungal growth on the pulp (*107,113,114*). However, the use of oxidative enzymes rather than whole organisms does avoid the problem of cellulose hydrolysis. *T. versicolor* produces laccase and MnP at the highest levels when the greatest increase in brightening occurs, Both laccase and MnP can reduce the content of lignin in pulp provided the conditions are correct (*107,113,114*).

Mnp requires hydrogen peroxide and Mn^{++} for full activity. Laccase requires oxygen and a low molecular weight compound, termed a mediator, for effective bleaching. Some pulps do not require addition of Mn^{++} (*115*). Either they already have sufficient Mn^{++} or chelators, such as oxalate, are able to liberate sufficient Mn^{++} for the enzyme to have an effect (*115*). As yet, no

natural mediators have been identified from the fungal cultures involved in bleaching, and some have thought that MnP is more important than laccase (*116*). Some evidence also suggests that extractives from Norway spruce might contain a natural mediator for laccase (*117*).

MnP and LiP both require hydrogen peroxide for activity, yet they are inactivated when it is present at moderately high levels. Both enzymes also contain heme, which must be inserted properly prior to secretion. Fungal cultures make these enzymes in relatively low yield. The cost of the enzymes, their requirements for hydrogen peroxide, and their sensitivity to hydrogen peroxide limited the early use of these enzymes (*114,118*). Enzymes are now more available, but their sensitivity to hydrogen peroxide still exists. Continuous low level addition of hydrogen peroxide (*116,118*), or a system to generate it (glucose oxidase and glucose), can provide the needed hydrogen peroxide (*115,119*).

The use of MnP and xylanase along with caustic extraction and hydrogen peroxide bleaching has been proposed as a TCF method of pulp bleaching (*118*). Hydrogen peroxide bleaching after MnP treatment has been found effective. In one instance, MnP increased the final brightness by 10 points (116). The simultaneous use of xylanase and MnP had a synergistic effect during a prolonged bleaching with continuous addition of hydrogen peroxide (*120*). The use of xylanase prior to laccase–mediatortreatment also allowed better removal of the lignin. However, the simultaneous treatment with laccase was ineffective (*121*).

As noted, the initial proposed use of laccase for bleaching (*122*) was limited by the availability of the enzyme and the cost of the mediators needed for lignin destruction (*114*). Laccase and MnP can both provide equivalent levels of bleaching (*113*), but laccase is much easier to produce in quantity and is commercially available. Both laccase and MnP cause an initial darkening of the pulp that is subsequently removed during bleaching and washing (*113*). The search for new laccases and new mediators has met with some success. New laccases (*123*) have been reported from various sources (*123,124*), which now include bacteria such as *Azospirillum lipoferum* (*125*) and *Bacillus subtilis* (*126*). New mediators are being tested (*123,127,128*) including transition metal complexes (*129*).

The more common laccase mediators, 1-hydroxybenzotriazole (1-HBT) and 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS), have been used by many researchers comparing effects with various laccases (*130-136*). 1-HBT and ABTS were better mediators for delignification than other mediators tested (*137*). However, both are expensive (*137*). Other mediators such as violuric acid can be used, but they suffer from inactivating the laccase as does 1-HBT (*138*). While laccase and mediators allow brightening of low lignin pulps, on high

lignin pulps, they still reduce the lignin content (*139*). However, the brightening effect is lost (*131*).

Different laccases have also been shown to react differently with mediators (*137,138*). Combinations have to be tested prior to use. A single treatment of pulp with laccase and mediators will only remove a percentage of the lignin (*140,141*). The extraction of the pulp with alkali, and a second treatment with laccase, will remove more lignin (*141*).

While there is great promise in using oxidative enzymes in the bleaching of pulp, the process is not yet economical. The cost of the enzyme will always be a factor. However, the sensitivity of MnP to hydrogen peroxide and the need for an inexpensive, effective, nontoxic mediator for laccase treatment are critical remaining problems.

Enzyme Use in Fiber Recycling

The primary objectives in recycling paper are to remove ink and other contaminants while retaining optical and strength properties of the fibers. Enzymes can be used to enhance dewatering (drainage) rates, facilitate contaminant removal, and increase bond strength in recycled fibers. Drainage resistance of secondary fibers adversely affects sheet formation, slows operation of the paper machine, and increases drying energy (*142*). Deinking and contaminant removal likewise can benefit from enzyme treatments that facilitate separation of fibers in the washing and flotation processes.

Refining and Drainage

When they are properly applied, endoglucanases can enhance the drainage rates for recycled fibers beyond what can be attained by polymer addition (*143-146*). Enzyme treatments require additional retention time and sufficient mixing to affect the fiber surface structure. At optimum pH, temperature, and consistency and with appropriate mixing, treatments can be achieved in as little as 15 minutes. The enzyme dose and treatment time depends on the preparation and its activity. Enzyme addition prior to refining can lower the energy required to meet strength specifications or can improve strength properties at a fixed refining level (*145*). Enzyme addition after refining increases freeness, thereby enabling operation of the paper machine at a faster rate (*146*). Enzyme-assisted refining can enable lower head box consistency for better paper formation, improved strength, decreased basis weight, and increased use of recycled fibers.

Mooney et al. (*147*) reported evidence indicating that increased drainage results from selective digestion of the smaller fiber fragments. However, this effect also could be attributable to removal of cellulose microfibrils from paper fiber surfaces (*148,149*). In either case, care must be exercised to keep treatments at a low dose because endoglucanases that attack amorphous cellulose also cause rapid loss of fiber strength (*150,151*). In an attempt to avoid strength loss, Pala et al. (*152*) examined the use of cellulose binding domains (CBDs) isolated from cellulase preparations following proteolytic digestion. At low doses, CBDs increased both drainage rates and paper strength properties, but at higher dosage rates, the beneficial effects on strength indices were less pronounced. Pala et al. (*152*) hypothesized that the beneficial effect on strength was attributable to an increase in the microfibrilation of the fiber surface. Increased drainage, however, was also attributable to residual hydrolytic activity because CBD preparations in which some reducing sugar release was detectable increased drainage to a greater extent than preparations without detectable activity.

The efficacy of enzyme treatment depends on the fiber type. Mechanical fibers are much more resistant than chemical fibers to cellulase activity. Low doses of cellulases can increase handsheet density and reduce coarseness while having minimal effects on strength. Such treatments are most effective with kraft fiber (*153*). Because bond strength restoration is most important with secondary fibers and because recycled fibers are often filled with calcium carbonate, alkaline cellulase activity might be a desirable characteristic (*154*). However, highly active alkaline cellulases have not yet been described.

Starches used in sizings can accumulate in the treatment water of fiber recycling mills and interfere with drainage. In such instances, alpha-amylase can improve drainage properties, presumably by reducing the viscosity of the backwater ***(255,256).*** Amylase treatments have been shown to increase the drainage of recycled paper pulp, allowing the paper machines to run faster ***(255).***

Recovered paper is recycled from various sources. Mixed office waste (MOW), old newspapers, and old corrugated containers (OCC) are the main recycled paper streams. The adhesives, glues, coatings, and binders present in recycled paper can cause the accumulation of stickies in the pulp slurry (*157*). When stickies accumulate into larger particles, they cause problems with the paper production and need to be removed from the system. An undefined esterase was reported to be effective in reducing the size and number of stickies in the recycled paper sources (*157*).

Cellulase and hemicellulase mixes have been reported to improve drainage of OCC pulps and liner boards and to allow a lower amount of polymeric additives to be used to strengthen the paper (*143,144,158*). The use of cellulases and xylanases can be a problem with anionic surfactants, which are often used

during recycling (*159*). The use of cationic or nonionic surfactants actually enhances the activity of cellulases and xylanases (*159*).

Deinking

One of the main applications of enzymes to recycled fibers has been to remove print (*160,161*). More than 70% of MOW paper consists of uncoated papers that are printed with copy and laser printer toners, which are often difficult to remove by conventional, alkaline deinking processes (162). Cellulases are particularly effective in facilitating the removal of toners from office waste papers (163). Both cellulase and a nonionic surfactant are required for effective deinking (164). Recently, Park and Park (165) showed that chemically modified cellulases were more effective for recycled fibers than unmodified cellulases. These researchers added polyethylene oxide copolymer derivatives and maleic anhydride to the amino groups of the enzyme and then used the modified enzyme to treat recycled fibers. Paper made from fibers treated with modified cellulase had better physical properties, such as tensile strength and internal bond formation, than fibers made from unmodified cellulase. The modified cellulase was more effective in separating ink particles from fiber during flotation deinking (*166*). Paper freeness, whiteness, and tensile strength increased by 31%, 13%, and 24%, respectively.

Treimanis et al. (*167)* showed that cellulase decreases the interaction of toner and ink particles with fibers. This treatment is more effective with alkaline sized papers after adjusting the pH to 3.0 and the addition of a hydrocarbon surfactant (*168*). Based on enzyme trials and microscopic observations, Dinus and Welt (*161*) concluded that the primary role of cellulases in deinking involves separating ink–fiber agglomerates and dislodging or separating ink particles and fibrous materials in response to mechanical action during disintegration. Alpha amylase also appears to have a positive effect on deinking of recycled fibers (*169*), but it is not clear whether this mechanism is the same as that observed with cellulase.

Other Enzyme Uses in the Pulp and Paper Industry

Deposit Removal

Water used for the paper process often contains nutrients and is kept in conditions conducive to microbial growth. Microbial growth or deposits can be a problem when mills have closed their water loops, providing the possibility of concentrating organisms and nutrients (170). The microorganisms that cause

these problems can be many different species, both aerobic and anaerobic (*170,171*). Slime formation on equipment is often the result of such conditions. Slime will cause many problems such as pump failure and breaks and blockages in paper machines. The first approach to this problem is to prevent slime formation by adding biocides. Once slime has formed, its removal is an important operation in the paper making industry and often requires shut down and caustic boil outs to properly clean the line.

Much of slime, or biofilm, is composed of carbohydrate and protein polymers. Enzymes such as amylases and proteases have been found useful in enzymatic boil out of slime encrusted equipment (*172-174*). One approach to the removal of slime from equipment is to produce sufficient slime from organisms isolated from the source, set up enrichments, and select organisms that will grow on it (*175*). Organisms were found that could degrade these polymers depending on the polysaccharide and organism used to make it. This approach may provide a source of new enzymes to be used for cleaning such deposits (*175*).

A similar approach was taken with colloidal material that could be reduced by culture filtrates of a fungus *T. versicolor* grown on the colloidal material (*176-178*). Dissolved and colloidal substances are a problem especially when mills close their water loops to conserve water use (179-181). Lipase has been used to remove lidid-based materials in these suspensions for a number of years (*182-186*). Removal of extractives by laccase-based catalysis of polymer formation and lipases provided additional removal of organic materials (*177*). Carbohydrate components of the colloidal material (*187*) could be removed by esterase treatment, which results in the carbohydrates being deposited onto the fiber (*28*).

Enzyme Use in Fiber Modifications

Interest has been growing in the enzyme-assisted modification of fibers. Enzymes such as peroxidases and laccases have been used to polymerize or co-polymerize materials with wood-based fibers (*188*). Attachment of guaiacol sulfonate using laccase made lignin more water soluble (*189*). The attachment of 4-hydroxyphenylacetic acid was also demonstrated, but no differences in lignin solubility could be distinguished (*189*). High kappa pulps were modified with 4-hydroxyphenylacetic acid in the presence of laccase (*190*). This modification increased the carboxylic acid groups, water retention, tensile strength, and burst strength of the resulting paper. The same treatment with tyrosine and guaiacol sulfonate had only minimal effects (*190*). Laccase catalyzed the polymerization of acrylic compounds onto lignosulfonates (*191*). Laccase in the presence of specific peroxides also grafted acrylamide onto lignin (*192*). Incubation of lignin with wood-based fibers in the presence of laccase formed covalent attachments with carbohydrate polymers (*193*). Laccase also

assisted in enhancing the bonding in fiberboard made from Norway spruce (*117*) and beech fibers (*194*). Water soluble components of the spruce thermomechanical pulp aided in this bonding, and if removed, a mediator could replace their effects (*117*). Mediators also improved the strength of unbleached kraft pulp modified with laccase (*195*).

Peroxidases and other oxidases have been used to modify polymers as well (*188*). Peroxidases have been used to attach gallate esters to chitosan (*196*). However, it was difficult to measure what reactions actually took place. Low molecular weight lignin fragments were precipitated by treatment with horseradish peroxidase or potato-polyphenoloxidase, thus removing them from solution (*197*). The use of manganese peroxidase in aqueous organic solvents allowed the polymerization of guaiacol and other phenolic compounds and aromatic amines (*198*). The ability to function in organic solvents and modify polymeric compounds allows many reactions to proceed via oxidative enzymes, since many of the substrates that the oxidative enzymes react with are sparingly soluble in water (*199*). While these enzymes are able to catalyze reactions efficiently, they operate by free radical addition, and the bond formation can be predicted but not necessarily controlled and directed. This is especially true when the polymer being used is complex like lignin and wood-based fibers.

Cellulases can increase fiber flexibility and thereby improve pulp properties. This can be particularly important for coarse fibers with thick walls such as those derived from Douglas-fir. An enzyme charge of 1 mg cellulase protein/g of ovendry pulp can improve handsheet density and smoothness while increasing freeness and reducing coarseness (*153*). Excessive enzyme treatment, however, erodes the fiber surface and reduces pulp strength (*200*). Degradation is most apparent with short fibers, and refining is least effective with the long, coarse fibers (*201*), so if the pulps are fractionated into various lengths prior to enzyme treatment, the results are more satisfactory (*202*). By treating the fiber fractions separately, each under optimal conditions, and then recombining the fractions, Mansfield and Saddler (*203*) were able to increase tensile strengths of the resulting pulps by 25% to 35%. Cellulase and xylanase treatments can increase the density of the resulting handsheets made from kraft pulps (*204*). The beneficial effect of cellulases and xylanases on pulp properties appears to result from a collapsing of the coarse fiber and a resulting increase in the surface area available for interfiber bonding (*201*). Mechanical pulps are much more resistant to enzyme treatment. Laccases can increase bonding of mechanical fibers, but this probably results from a different mechanism than that observed with cellulases and xylanases (*25*).

Cellulose Binding Domains

Enzymes that attack cellulose and xylan often have portions (domains) of the protein that bind to cellulose (*205,206*). Binding domains specific for xylan have been identified (*207*), but a single amino acid change can alter this specificity from xylan to cellulose. Xylanase catalytic subunits with CBDs as part of their overall tertiary structure are common. The biochemical role of a CBD is to keep the enzyme catalytic unit close to the substrate surface. The need for the CBD in fiber processing might depend on the uses of the enzyme. The roles of CBDs in the hydrolysis and modification of chemical pulps have been examined (*208*). The presence or absence of CBDs did not affect the action of enzymes against soluble substrates. However, enzymes with a CBD enhanced fiber beating to a greater extent than did enzymes without the CBD. Since beating is thought to increase microfibrillation, the CBD could play a role in opening up the cellulose crystallite structure.

Opening the structure of cellulose or swelling of fiber has recently been attributed to swollenin (*209*), a protein made by *Trichoderma reesei* that has homology to CBD and expansin (*210*). The latter is a plant protein involved in separating cellulose fibrils during wall expansion and cell growth. Thus, CBD might be viewed as a domain that makes substrates more accessible (*210*). The recombinant-derived CBD of *Cellulomonas fimi* actually blocked the access of cellulase to the substrate rather than making it more available (*210*). Deletion of the CBD of xylanases from *Pseudomonas fluorescens* and *C. fimi* did not affect the ability of the enzymes to attack pulp xylan but had variable effects on reducing the lignin content and bleaching (*211*). The *P. fluorescens* enzyme had the same effect on bleaching with or without the CBD, whereas the *C. fimi* enzyme was marginally better with the CBD. Similar findings on the need for the carbohydrate binding module for the hydrolysis of insoluble xylan by the xylanase from *Clostridium stercorarium* have also been reported (*212*). The xylanase might have dual functionality being both a cellulose binding protein and a xylanase (*213*).

Some proteins with an ability to bind to cellulose do not have a specific definable CBD. The cellobiose dehydrogenase from *P. chrysosporium* binds less strongly than the cellobiohydrolase of *T. reesei* (*214*). Attempts to localize a CBD from the cellobiose dehydrogenase via proteolysis indicated that the structure is probably internal to the structure of the protein. The proteolysis of a cellulase complex from *Penicillium verruculosum* provided a fraction that was able to hydrolyze soluble xylan but did not hydrolyze insoluble xylan (*215*). The xylanase from this organism was a major component of the cellulase complex and presumably contains a strong CBD (*215).* New CBD sequences are being discovered, and there may be multiplicity (*216*) in the binding of carbohydrases to the substrates they attack.

Some of the more interesting uses of CBDs might be to alter fiber surfaces without actually hydrolyzing the fiber. For example, Kitaoka and Tanaka (*217*) have recently described a CBD-based additive that enhances paper strength.

Modifying Enzymes to Attain Activity Under Specific Conditions

The cloning of specific enzymes from described organisms and DNA sequences is becoming routine. Many new xylanases (*34,50-55,67,68,218-232*), cellulases (*50,51,53-55,233,234*), and other fiber modifying enzymes (*50,53-55,92,235,236*) have been recently cloned, produced, and characterized as to their activities. Often these enzymes can be produced without ever having to deal with the natural host. With the plethora of genomic sequences being discerned, many new enzymes might be available by searching the new sequences for regions that code for specific enzymes. This directed approach has provided new potential enzymes for use.

Degenerate primers coding for conserved regions of enzymes have been used in PCR studies to produce gene fragments encoding these enzymes. This technique has been used to produce novel enzymes from unknown organisms present in the environment. A recent example of this is the cloning of a 2,5-diketo-D-gluconic acid reductase from an unknown organism (*237*). The resultant enzyme had much higher catalytic activity than existing enzymes from known organisms. Although there may be some limitations on what might be made by recombinant organisms, there are many enzymatic targets that might benefit from this approach. Thermophilic organisms must have active enzymes for all of their cellular functions. The nature of the environment in which these organisms are found dictates that these enzymes must be thermostable. Many enzymes have been cloned from the DNA of these organisms. Although a thermophilic enzyme can tolerate higher temperatures, it will act slower at lower temperatures. Thus, a thermophilic enzyme should be sought when a process has to remain hot or requires the use of solvents, but many other alternatives exist when the process does not have to be heated and is at a nondenaturing pH. In fact, using a thermophilic enzyme might cost more for a given treatment if the fiber has to be specifically heated for the enzyme to be effective.

When an enzyme has been studied to the extent that the crystal structure is known, some specific planned changes can be made and the response in the activity of the protein predicted. The introduction of disulfide bonds through site-specific mutations have increased the thermostability of a *B. circulans* xylanase (*238*). All of the designed changes provided increased thermostability. However, only one out of eight changes provided an increase in the optimal temperature of activity (*238*). Site-specific changes were also introduced into the MnP of *P. chrysosporium*, which allowed the enzyme to tolerate higher

concentrations of hydrogen peroxide than the wild type (*239*). The increased tolerance was somewhat offset by a decrease in the affinity of the enzyme for hydrogen peroxide.

Site-specific changes require knowledge of the sequence of the protein and some concept of how changes can be imparted. Comparison of the sequence of *Streptomyces lividans* xylanase to thermostable xylanases from the same family provided a road map to increasing the thermostability of the *S. lividans* enzyme (*229*). The expression of xylanases in *E. coli* and a screening assay for thermostability also helped to identify more thermostable variants of the *S. lividans* xylA xylanase (*221*). Domains that have thermostabilizing activity have been identified (*33,240*). The first 29 amino acids of the *Thermomonospora fusca* xylanase A enzyme imparted increased thermostability to *S. lividans* xylanase B (*241*) and also *T. reesei* xylanase II (*242,243*). An increase in optimal operating temperature was found in variants of *S. lividans* xylanase B by further random gene shuffling using random fragmentation of the *T. fusca* and *S. lividans* genes (*241*). The thermostability and thermal activity of the modified *T. reesei* enzyme has been incorporated into the BioBrite HB60C, which shows improved activity in ECF bleaching (*242,244*).

Other domains from the family 10 xylanases have also been shown to have thermostabilizing effects. Domains from *C. fimi* (*227,245*), *Thermomonospora alba* (*218*), *Neocallimastix frontalis* (*228*), *Streptomyces olivaceoviridis* (*218,245*), and *Fibrobacter succinogenes* (*246*) have all been studied. The results from these studies indicate that the domains often convey their function to new chimeric proteins. This could be the ability to bind to cellulose, bind to other proteins (*246*), change active site domains, and change substrate binding domains (*245*). The shuffling of modules or domains can again produce proteins with altered characteristics. An example of modified enzyme would be the combination of cellulase activity with that of (1-3,1-4)-b-glucanase activity (*247*). This multienzyme was able to degrade the specific linkages present in barley glucan.

The altering of an enzyme using random mutations is powerful and does not require detailed structural information. The *Coprinus cinereus* peroxidase was modified using both site directed and random methods (*248*). The manual combination of individual site directed mutants resulted in an enzyme with 110 times the thermal stability and 2.8 times the oxidative stability. However, when ***in vivo*** shuffling of mutations through yeast homologous recombination was used, an enzyme with 174 times the thermostability and 100 times the oxidative stability was obtained (*248*).

The directed evolution of an enzyme can provide a powerful tool to attain enzyme optimization. The technique requires a useful host organism and a screen to select desired clones (*249*). Directed evolution increased the alkaline activity and thermostability of a *Neocallimastix patriciarum* xylanase (*250*). Error prone PCR, mutator strains, DNA shuffling, and domain swapping can all be used when the selection is powerful and specific (*249*). Clearly, knowledge

and random changes can be applied, and this allows nature to provide a range of possibilities for enzyme modification.

In silico design of an enzyme may lead to further development in this area (*251,252*). Intimate structural knowledge of an enzyme's active site is required for these types of modifications. However, the alterations provided by nature are rather finite and work within a context of what was already present and working, With an enzyme designed by in silico methods, the principles of protein folding, bond angles, hydrophobic cores, hydrogen bonding solvation, and other forces (*253,254*) can provide us with so many possibilities (many of which have not been produced by nature) that they cannot be all tested. Designing thermophilic variants of proteins with this technology is already possible (*255*). The temperature limit for enzyme activity might be as high as 200°C (*256*). Narrowing these possible structural variants to what can be readily tested is the challenge (*257*).

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