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4 Fungal enzymes for lignocellulose degradation

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4.1 **Introduction**

Microbial degradation of lignocellulose has been intensively studied for decades. Interest in lignocellulose as a renewable resource prompted these investigations, although in recent years fungal enzymes have attracted much attention in pulp and paper manufacturing. Irrespective of considerable research effort, fundamental questions remain with regard to mechanisms of lignocellulose degradation. Progress has been hampered by numerous factors including substrate complexity and the multiplicity of enzymes.

Not surprisingly, this intense interest has resulted in voluminous literature. In this chapter literature relevant to lignocellulose degradation by filamentous fungi is outlined. The format of the text is organized around substrates of increasing complexity (cellulose, hemicellulose, and lignin) and recent developments are highlighted. Areas where knowledge is sketchy are identified. Except as points of reference, prokaryotic systems are not discussed, and the reader is referred to numerous review articles for additional information.

4.2 **Cellulose degradation**

Cellulose is the most abundant renewable organic resource. It comprises approximately 45% of dry wood weight. Crystalline cellulose is composed of long β- chains (*c.* 40) that are hydrogen bonded into water-insoluble microfibrils. The crystalline cellulose coexists with less ordered amorphous regions. The fibers are embedded in a matrix of hemicellulose and lignin, but the precise organization is poorly understood [1]. Cellulose is often characterized by the degree of polymerization (D P), which is the average number of glucan residues per molecule. The average DP of native cellulose is 3500 [2].

Bacterial and fungal degradation of cellulose has been extensively studied and reviewed [3– 10]. The soft-rot fungi, *Trichoderma viride* and *Trichoderma reesei* are, by far, the most extensively studied. During the past few years, these investigations have made significant progress toward elucidating the enzymology of cellulose degradation. Among prokaryotes, the Corynebacterium *Cellulomonas fimi* and the anaerobe *Clostridium thermocellum* are the best known examples.

All cellulase systems share common features. However, the mechanism of fungal brown-rot decay is clearly different from other systems, and the multicomponent enzyme complexes of some bacteria such as *C. thermocellum* are also distinct. Oxidative enzymes of fungi may also participate in cellulose degradation. This chapter focuses on recent advances in the enzymology of hydrolytic cellulases of fungal origin. in the molecular genetics of these fungi, and in the potential biotechnological applications.

4.2.1 *Cellulase biochemistry*

In submerged culture, most cellulolytic fungi secrete a complex array of degradative enzymes. Three classes of hydrolytic cellulases are recognized on the basis of substrate specificity:

(i) Endo- **1,4-β-glucanases** (EG) cleave randomly at **1,4-β-linkages** within the cellulose chain. The endoglucanases are commonly assayed by viscosity reductions in carboxymethyl cellulose (CMC) solutions. Crystalline cellulose is not degraded by endoglucanases. Multiple chromatographically distinct endoglucanases have been identified in culture filtrates of *T. reesei [8]* and *Phanerochaete chrysosporium* [11].

(ii) **Exo-1,4-β-glucanase** (exo- **1,4-β-D-glucan** cellobiohydrolases, CBH) of *P. chrysosporium,* releases both glucose and cellobiose from the non-reducing ends of cellulose chains [12]. In contrast, the two cello biohydrolases of *T. reesei* split off cellobiose only. Cellobiohydrolases have virtually no activity on CMC and slowly degrade crystalline cellulose. Both endoglucanases and cellobiohydrolases are active on amorphous (non-crystalline) cellulose.

(iii) **1,4-β-glucosidases** hydrolyse cellobiose to glucose, and cellobionic acid to glucose and gluco-nolactone. Two *P. chrysosporium* enzymes, one cell-bound and one extracellular, have been identified [13]. Relative to endoglucanases and cellobiohydrolases, low levels of the *T. reesei* **β-glucosidase** are secreted in submerged culture. For certain applications, supplementing *T. reesei* cellulase preparations with additional **β-glucosidase** has proved beneficial [14, 15].

Fungal cellulases have frequently been reported to act synergistically in the degradation of crystalline cellulose. A commonly held model for the synergism between endoglucanases and cellobiohydrolases suggests that hydrolysis is initiated by endoglucanases that cleave random β- 1,4-linkages within the cellulose chain. This activity is followed by celiobiohydrolase action that releases cellobiose from the non-reducing ends of the cellulose chain (reviewed in [3, 16]). Studies with *T. reesei* suggest a more complicated interaction [17] in that the extent of synergism may be related to the substrate used and the ratio of cellobiohydrolase to endoglucanase.

The discovery of synergism between two immunologically distinct

cellobiohydrolases [CBHI and CBHII) from both *T. reesei [18]* and *Penicillium pinophilum [19]* indicates that the 'endo-exo' model is incomplete. A model for 'exo–exo' synergism, which is based upon substrate stereospecificity, has been advanced by Wood and McCrae [19]. Recent evidence demonstrates different stereochemical mechanisms for *T. reesei* CBHI and CBHII [20, 21].

Attempts to formulate a model of cellulose degradation have been stymied by uncertainties concerning the exact roles and specificities of *P. chrysosporium* and *T. reesei* cellulases (reviewed in [4]). For example, highly purified *T. reesei* endoglucanase has been reported to be active only on soluble β- 1,4-glycans [22]. In addition, studies of purified *T. reesei* CBHI have demonstrated that the enzyme has endoglucanase-like properties including the ability to digest barley β-glucan [17] and to bind along the length of cellulose microlibrils [23]. To further complicate the issue, *T. reesei* cellobiohydrolase has been reported to be capable of degrading crystalline cellulose extensively, without assistance from any endoglucanase activity [24].

The wealth of conflicting results in the field of cellulose degradation [4] clearly calls into question the purity of many enzyme preparations that have been studied. Wood and co-workers [25] have demonstrated endoglucanase/cellobiohydrolase complexes that were resolved only after affinity chromatography [26]. When these highly purified enzymes were used to degrade microcrystalline cellulose in the form of cotton fiber, three enzymes (CBHI. CBHII, and trace endoglucanase) were necessary for rapid hydrolysis. Clearly, enzyme purity must be carefully established for studies of enzyme synergism.

Recombinant enzymes produced in heterologous hosts provide a powerful tool for resolving the uncertainties of cellulase substrate specificity. The finding that recombinant *T. reesei* CBHI lacked any β-glucanase activity [27] demonstrates the important role cellulase expression systems can play in answering such questions.

The mechanism of cellulose degradation by brown-rot fungi is poorly understood, but some progress has been made in recent years. Wood decay by brown-rot fungi such as *Poria placenta is* characterized by very rapid depolymerization of cellulose (reviewed in [3]). A large molecular weight multifunctional endoglucanase has been identified in culture filtrates but no exoglucanase has been found [28]. Formation of the enzyme is not repressed by glucose [29]. Perhaps the most striking feature of the brown-rot systems is that cellulose depolymerization appears to involve a non-enzymatic, diffusible agent. An oxidative mechanism is suspected although the exact agent is unknown [30].

4.2.2 *Molecular genetics and structure of fungal cellulases*

The molecular genetics of cellulolytic fungi, especially *T. reesei,* has advanced considerably over the past five years. Research has revealed extremely

interesting structural features of the cellulase gene family that have important implications with regard to their mechanism(s) of action and evolution. From *T. reesei,* sequences have been reported for both genomic and cDNA clones encoding *cbh1 [31,* 32], *cbh2 [33, 34], egl [35], eg3 [36]* and β-glucosidase [37]. Other sequenced fungal clones include *P. chrysosporium chb1 [38]* and *Humicola grisea* var. *thermodea cbh1 [39].* An *Aspergillus niger* gene encoding β-glucosidase has been cloned [40].

Several lines of evidence indicate that cellulases are made of three structural regions: the catalytic 'core' which hydrolyses β- 1,4-glycosidic linkages, the extended 'hinge' which is glycosylated, and the cellulose-binding 'tail' which has also been hypothesized to help solubilize the cellulose chain prior to hydrolysis (reviewed in [8,41]). Comparisons of amino acid sequences of *T. reesei cbh1, cbh2. eg1,* and *eg2* have shown the presence of two short consecutive blocks that are conserved in all four genes (Figure 4.1). Block A is approximately 30 amino acids long and is stabilized by 2 or 3 disulfide bridges. Sequence conservation is high within block A (c. 70% identical). Block B is approximately 40 amino acids long and is heavily glycosylated with > 40% serine, threonine. or proline. Two copies of block B are present in *cbh2.*

Another line of evidence for functional domains is through extensive analyses of core and terminal domains prepared by limited proteolysis with papain [42]. The catalytic activity [43, 44], crystalline cellulose-binding capacity [42, 44, 45], and structure [46–48] of these cleavage products support the general model. T. *reesei* EGIII also requires the conserved blocks for efficient binding and hydrolysis of crystalline cellulose [49]. The functional domains can also be separated by native proteases in cellulolytic bacteria [50] and fungi [49,51].

Based upon small angle X-ray scattering analysis [47. 48] and NMR spectroscopy [46], a 'tadpole-like' tertiary structure has been proposed for CBHI and CBHII (Figure 4.2).

A two-functional domain is a common design of fungal and bacterial cellulases. For example. Gilkes and co-workers [50] have separated and

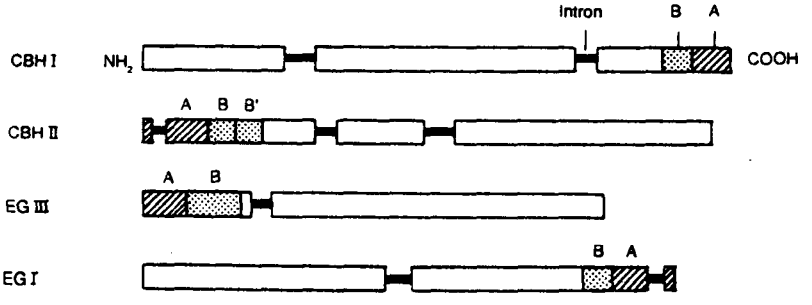

Figure 4.1 Representation of *T.* reesei cellulases. Microcrystalline binding regions and glycosylated hinge shown as blocks A and B, respectively. (Adapted from Knowles and colleagues [27].)

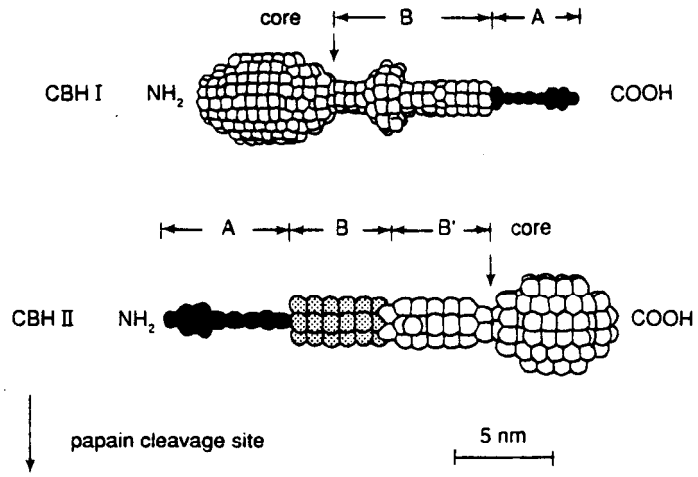

Figure 4.2 Model tertiary structure of *T. reesei cellobiohydrolase.* As in Figure 4.1. the binding domains and hinge region are designated A and B. respectively. (Adapted from Abuja and colleagues [47].)

characterized the catalytic and binding domains of *C.fimi* endoglucanase and exoglucanase. The domain structure has also been experimentally demonstrated in other bacteria including *C. thermocellum, Thermononospora fusca* and *Bacteroides succinogens* (reviewed in [4. 5]) as well as the fungi *P. chrysosporium [52]* and *P. pinophilum [41].*

Stahlberg and co-workers [49] have studied the kinetics of adsorption to crystalline cellulose by the isolated domains and by intact CBHI [43]. They propose a model in which the enzyme binds at two points (core and binding domains) and laterally diffuses on the crystalline surface.

Recent information has been gained concerning the active sites of cellulases. X-ray crystallography of the CBHII catalytic core suggests a tunnel-shaped active site [53, 54]. Consistent with studies of cellobiohydrolase degradation of cello-oligosaccharides [41, 55], four glucosyl binding sites have been identified within the tunnel. Cleavage of the cellulose chain is likely to occur within the tunnel between aspartic acid residues 175 and 221. Comparisons of amino acid sequenccs of CBHII and endoglucanases show deletions in the endoglucanases that would result in a more open active site or 'groove'. Rouvinen and co-workers [54] propose that these structural features may account for substrate specificities. The exoglucanases hydrolyse the ends of chains threaded within the tunnel, whereas the open grooves of endoglucanases can hydrolyse internal glycosidic bonds [54].

Hydrophobic cluster analysis has been used to classify 21 β-glucanases and to identify potential catalytic residues [56]. The six fungal cellulases included in the analysis were categorized into three of the six families. Only family A contained sufficient numbers of sequences to allow tentative identification of

catalytic residues. These correspond to His-174 and GIu-218 of mature *T. reesei* EGIII.

Catalytic sites have also been tentatively identified by chemical modifications of cellulase. Tomme and Claeyssens [57] have implicated a Glu- 126 in CBHI catalysis. Lending support to this is hydrophobic cluster analysis of EGI and CBHI, which shows considerable sequence conservation in the Glu- 126 region [56]. Chemical modifications of *Schizophyllum commune* EGI have shown the importance of carboxyl groups in catalysis [58]. This, plus some weak sequence homology between T4 bacteriophage lysozyme and certain fungal cellulases, have suggested similar catalytic mechanisms [51. 59]. However, the putative catalytic *S. commune* EGI residues, Glu-33 and Asp-50. are not conserved in the *T. reesei* EGIII [36]. The tertiary structure of CBHII is also inconsistent with the lysozyme model [54].

In contrast to studies of cellulase structure and mechanisms, relatively little work has been done with regard to their regulation. The cellulases of *T. reesei* and *P. chrysosporium* are coordinately induced in media containing cellulose or derivatives (e.g.. sophorose. cellobiose) as carbon source. The effectiveness of the inducers varies substantially with the particular disaccharide and the species. Thus, sophorose is a relatively poor inducer in *P. chrysosporium [60],* but very efficient in *T.* reesei. Enzyme synthesis is end-product inhibited by glucose. although low levels of *T. reesei* CBHII have been detected in glucose medium [61]. In the case of *T. reesei* and *P. chrysosporium,* induction has been shown to occur at the transcriptional level [34. 38].

The precise mechanism(s) of cellulase induction is unknown. It has been suggested [62] that low level. constitutive expression of cellulases might mediate the induction by producing low molecular weight, soluble compounds that could enter the cell and cause induction. Direct experimental evidence for this mechanism in *T. reesei* has been demonstrated by using cellulase-specific antibodies to block CBHI induction by cellulose [63]. The repression by antibodies could be overcome by sophorose induction [63]. The identity of the 'natural' inducer is still uncertain. although β-linked disaccharides are likely. Frischer and co-workers [64] emphasize the importance of β-glucosidase in the induction process.

In addition to the hydrolytic enzymes described thus far, several oxidative enzymes have been implicated in cellulose degradation by *P. chrysosporium.* These include cellobiose oxidase [65] and cellobiose: quinone oxidoreductase (CBQase) [66]. Cellobiose oxidase may stimulate cellulolytic activity by reducing cellobiose concentration. which appears to be a competitive inhibitor of cellulases, and by oxidizing reducing ends formed by endoglucanases thus preventing reformation of β- 1.4-glycosidic bonds [65]. CBQase may be an important link to lignin degradation by reducing quinones while oxidizing cellobiose. However, recent work has shown that CBQase does not affect phenoxy radicals produced by lignin peroxidase or phenol polymerization catalysed by lignin peroxidases *in vitro* [67]. Lactonases may be important in

cleaving the saccharide-lactone products of CBQase [68]. Both enzymes have been shown to bind microcrystalline cellulose while retaining enzymatic activity [69]. Recently, Henriksson and co-workers [70] have demonstrated papain cleavage of cellobiose oxidase into two domains: one containing flavin and the other containing a heme group. The flavin-containing domain binds crystalline cellulose and is functionally similar to CBQase leading Henriksson and co-workers to suggest that CBQase forms may be derived from cellobiose oxidase through proteolysis. Two acidic proteases have been identified in *P. chrysosporium* culture filtrates [71], although their specificities are unknown.

Relative to well-defined genetic systems, such as *Saccharomyces cerevisiae, Neurospora crassa,* and *Aspergillus nidulans,* genetic analysis of cellulolytic fungi has been extremely limited. *Trichoderma, Penicillium,* and *Humicola* species lack any meiotic or mitotic (parasexual) recombination system. Methods of genetic analysis are well established for *S. commune,* but the cellulases have not been studied. The mating behaviour of *P. chrysosporium is* somewhat controversial [72, 73], although it is clear that sexual crosses and meiotic recombination are possible [73, 74]. Importantly, there appears to be general agreement that the basidiospores of *P. chrysosporium,* although binucleate, are homokaryotic. Analyses of single basidiospore cultures have been used to determine allelic relationships [75, 76] and to generate a genetic map using restriction fragment length polymorphisms (RFLP) [77]. Linkage between a *cbhl* gene [38] and a lignin peroxidase gene has been established using the RFLP map [77, 78]. Recently developed transformation systems for *T. reesei* [79], *P. chrysosporium [80,81],* and *S. commune [82]* have opened the way for more detailed genetic analyses.

4.2.3 *Applications*

Enzymatic saccharification of lignocellulose and subsequent fermentation to ethanol have been studied for many years (reviewed in [3]). A major objective of these studies has been to express the cellulase genes in heterologous systems. The *T. reesei* cDNAs encoding CBHI [83], CBHII [84], EGI [83, 85] and EGIII [27] have been expressed in *S. cerevisiae.* In each case. highly expressed yeast promoters were used, and 10– 100 mg of heavily glycosylated product were obtained per liter of fermentation broth. The approximate native molecular weight was restored after treatment with glycosidase endo H [85, 86], indicating that giycosylation was N-linked. In one case, glycosylation appeared to confer thermostability [85]. Expression of *T. reesei eg2* in brewers' yeasts shows promise for reducing barley β-glucans [86]. Bacterial expression systems have not proven useful for *T.* reesei cellulases. Beguin [5] has reviewed expression of bacterial cellulase genes in heterologous hosts.

Cellulase expression has been altered in *T. reesei.* Strains lacking CBHI and others with increased EGII yields have been engineered [87]. Recently, Barnett and co-workers [37] have improved *T. reesei* cellulose saccharification rates

by increasing β-glucosidase expression. The β-glucosidase gene was cloned, sequenced, and multiple copies re-introduced to *T. reesei*. One such transformant exhibited a five-fold increased rate of cellobiose hydrolysis.

Protein fusions involving cellulase-binding domains may provide an inexpensive and widely applicable method for immobilizing and purifying enzymes. In one case, the *Agrobacterium* gene encoding β-glucosidase was fused in frame to the cellulose-binding domain of an exocellobiohydrolase from *C. fimi*. The translational fusion was expressed in *E. coli*. When immobilized onto cellulose, 42% of the β-glucosidase activity was retained [88]. The immobilized enzyme is stable for long periods but can be desorbed at low ionic strengths or at alkaline pH [89]. Similarly, the cellulose-binding domain of the *C. fimi* endoglucanase was fused to alkaline phosphatase [90]. Although the approach to date has been limited to bacterial cellulases and *E. coli* expression systems, it is clear that the binding domains of fungal cellulases and fungal expression systems could be similarly exploited. Given the efficiency of heterologous expression in filamentous fungi such as *T. reesei* and *Aspergillus* spp. (chapters 5 and 6), relatively higher yields of fusion proteins might be expected.

An important application involving lignocellulolytic fungi is biomechanical pulping, which is defined as the use of fungi as a pretreatment for wood chips prior to mechanical pulping. Initial research on biomechanical pulping has been limited, but studies in several laboratories have shown that the fungal pretreatment could significantly decrease refining energy and enhance pulp strength properties [91 –94]. Two studies have examined the effects of fungal treatment on crude mechanical pulps instead of on wood before pulping [95, 96]. The process has been refined in more recent studies [97-100].

A fundamental requirement for efficient biopulping performance is the elimination or reduction of cellulase activity. Towards this goal mutants of *P. chrysosporium* exhibiting altered cellulase secretion have been isolated [101, 102]. An ultraviolet-generated Cel⁻ mutant, cel 44, has been intensively studied [103]. Treatment of wood chips with cel 44, prior to refiner pulping, reduced pulping energy requirements and increased strength properties of the resultant paper. However, such mutants are genetically undefined and pleiotropic effects such as lowered phenol oxidase production were noted [103]. This result is not surprising considering the mutagenesis procedure and the multiplicity of cellulase genes in *P. chrysosporium* [104].

In the absence of efficient Cel⁻ mutants, glucose impregnation of chips before fungal inoculation has been necessary. The relative importance of glucose as a repressor of cellulases rather than as a source of easily assimilated carbon is unknown. However, it is clear that pretreatment with wild-type strains, in the absence of glucose repression, will lead to excessive cellulose degradation.

In summary, the exact roles and specificities of fungal cellulases are not clearly understood. This is particularly true in complex substrates such as

wood chips. Further, the relationship between lignin and cellulose degradation is also poorly understood, although it is generally believed that cellulolytic and hemi-cellulolytic activity, as opposed to ligninolytic, provides most of the assimilated carbon for growth. This issue, the role and interactions of individual cellulases, is of paramount importance not only to understanding the mechanism of wood degradation but also to the successful development of processes such as biomechanical pulping.

4.3 Hemicellulose degradation

In contrast to cellulose, which contains only 1,4-β-glycosidic linkages, hemicellulose is structurally more complex. Accordingly, the enzyme systems giving complete degradation to monomeric sugars are similarly complex. Relative to cellulases, little is known concerning the molecular genetics of hemicellulases or their structure/activity relationships. However. research efforts have intensified in recent years because the hemicellulases have shown promise in pulp and paper production.

Hemicellulose is second only to cellulose in abundance. with dry wood concentrations ranging from 20 to 30%. Hemicellulose has three basic forms: 1,4-β-D-xylans, 1,3- and 1,4-β-D-galactans, and 1,4-β-D-mannans. The relatively short chains (average DP < 200) are frequently branched and the sugar residues are often acetylated or methylated. The chemistry and structure of hemicellulose have been reviewed [105–109]. Its biosynthesis is distinct from cellulases [110].

Hemicellulose is found in both secondary and primary walls and the composition varies substantially among species. Galactoglucomannans and arabinoglucuronoxylans are major components of softwoods. Glucuronoxylans, and to a lesser extent glucomannans. dominate in hardwood species. The arabinoglucuronoxylans and the glucuronoxylans, collectively referred to as xylans, represent approximately 7–12% and 15–30% of softwoods and hardwoods. respectively [2].

An important issue with respect to biotechnical exploitation of hemicellulases is the nature of Iignin-hemicellulose bonds. Ester and ether linkages occur between hemicellulose sugar hydroxyls and the *x-carbonyl* of phenyl propane subunits of lignin (reviewed in [111]). Evidence suggests that difficulties in lignin removal during pulping processes are due to these bonds [112].

4.3.1 *Enzymes*

The complete hydrolysis of hemicellulose into monosaccharides requires the concerted action of several enzymes. These include β-D-xylanases, β-D-galactanases, β-D-mannanases, as well as glycosidases β-D-xylosidase, *x*- and

β-D-galactosidase, and β-D-mannosidase. Esterases also participate by hydrolysis of acetylated carbohydrates.

A large number of filamentous fungi, yeasts. and bacteria are reported to produce 1.4-β-D-xylanases [3, 113]. These enzymes have been subdivided by their ability to release L-arabinose from arabinoxylans and from arabinoglucuronoxylans [114]. Arabinose-releasing xylanases are 'de branching' as opposed to the more common 'non-debranching' enzymes that do not liberate arabinose from the same substrates. Both types are capable of attacking unsubstituted 1,4-β-D-xylans and glucuronoxylans. Debranching and non-debranching 1,4-β-D-xylanases have been identified in cultures of *A. niger* [115] and *Ceratocystis paradoxa* [116, 117]. The xylanases can be further subdivided by a variety of physical characteristics (molecular weight (MW), *pI,* pH optima) which have been tabulated [3, 114].

Regulation of xylanase production has been studied in several fungi, virtually all of which also produce cellulases. In *Trichoderma* spp., sophorose and lactose induce both enzyme systems [118, 119]. However, separate control of cellulases and xylanases is observed with other substrates such as xylan and xylobiose. Like the cellulases, glucose repression of xylanase is common [119, 120].

The multiplicity of xylanases, especially those from *Aspergillus niger, Trichoderma* spp., Bacillus spp., and *Streptomyces* spp., has raised fundamental questions concerning their origin and function. Wong and co-workers [121] note a conserved relationship between molecular weight and *pI* when xylanases from the different species are compared. That is, low molecular weight xylanases tend to be basic whereas high molecular weight is associated' with more acidic *pI.* A notable exception to this relationship is the *S. commune* xylanase (M W, 22000; *pI* 4) [122. 123], but the relationship is conserved *even* in yeast-like fungi that lack multiple xylanases, e.g., *Cryptococcus [124–* 126]. Conservation of this relationship and observed differences in the activities of xylanases suggest distinct functions for the multiple xylanases.

Uncertainties remain concerning the roles and interactions of 1,4-β-D-xylanases in hemicellulose hydrolysis. Progress has been hampered by the complexity of natural substrates. the difficulties and expense associated with synthesis of model oligosaccharides. the problems inherent in preparing highly purified enzymes, and the lack of standardized assays. These issues have been reviewed by Erikkson *et al.* [3] and Wong *et al. [121].*

Recent progress has been made towards production of recombinant xylanases. The xylanase gene of *Clostridium thermocellum (xynZ)* has been cloned, sequenced, and expressed in *E. coli [127,* 128]. Interestingly, the 5', non-catalytic region of the gene shares significant homology with endoglucanases from C. *thermocellum,* suggesting the possibility of conserved binding domains. The amino acid sequence within the catalytic region is 43% identical to the exoglucanase of *C. fimi,* which is reported to have xylanase activity [129]. The xylanase gene of *Streptomyces lividans* has also been cloned [130]

and several *Bacillus* xylanase genes have been cloned and expressed in *E. coli [131,* 132]. The xylanase clone from *Butyrivibrio fibisolvens [33]* shows considerable amino acid identity with another *Batiks xylanase [134].*

To our knowledge there have been no reports of xylanase clones from filamentous fungi. This is somewhat surprising given the variety and quantity of xylanases produced by filamentous fungi and the apparent conservation of amino acid sequence [122, 123]. As the following describes, recent interest in the use of xylanases for pulp and paper processing will inevitably change this situation. Both cDNA and genomic clones encoding the xylanases of *Collerotrichum albidus,* a yeast-like fungus, have been sequenced [135].

The complete breakdown of xylans requires hydrolysis of xylo-oligosaccharides to xylose by xylosidases. Most β-D-xylosidases show little or no activity toward xylans and hydrolysis rates decrease with increasing length of xylo-oligosaccharides [136–138]. Additional activities such as glycosyltransferase have been ascribed to several β-D-xylosidases [138–141]. The question of xylosidase specificity has been reviewed [3, 142]. Among the many fungi surveyed [142], xylosidase production was inducible by methyl-β-D-xylopyranosidase. The enzymes are primarily intracellular.

Several additional enzymes participate in xylan breakdown by cleaving side chains. α-L-arabinofuranosidases hydrolyse the α-1,3-linked arabinofuranosyl side chains of arabinans, arabinoxylans. and arabinogalactans [143–145]. *x*-D-glucuronidases cleave the 4-*O*-methyl glucuronic substituents from xylan chains [146]. Biely and co-workers [147, 148] purified and characterized acetyl xylan esterase from several fungi. Evidence supports synergistic relationships between these enzymes and the 1.4-β-D-xylanases [148, 149]. At least one fungus, T. *reesei,* produces all of these enzymes [150].

Enzymatic degradation of mannan components of hemicellulose involves 1,4-β-D-mannanases. β-mannosidases. β-glucosidases. and α-galactosidases. Dekker [114] and Erikkson and co-workers [3] have reviewed the mannan-degrading enzymes.

4.3.2 *,Applicalioms*

The enzymatic conversion of hemicellulose hydrolysates to monomeric sugars and subsequent fermentation to ethanol have attracted attention for many years [151]. Although hexose derivatives are efficiently utilized by *S. cerevisiae,* xylose fermentations have been problematic. Several yeast-like fungi, notably *Candida tropicalis, Pachysolens tannophilus* and *Pichia stipitis.* have been shown to ferment xylose. but considerably more work is needed to improve the efficiency of the fermentations [152]. *S. cerevisiae cells* transformed with xylose reductase and xylitol dehydrogenase genes of *P. stipitis* will utilize xylose as the sole carbon source [153]. However, these transformants utilize xylose oxidatively, and ethanol yieids are very low.

Hemicellulases, particularly, 1.4-β-D-xylanases, are becoming increasingly important in the treatment of pulps. Many studies have focused on reducing or eliminating the oxidative chlorine extractions that are used to bleach kraft pulps. The impetus for these efforts has been largely due to environmental concerns over discharge of chlorinated organic compounds, especially dioxin. The industry's scale is worth noting. Approximately 48 x 10^6 tons of kraft pulp were produced in the US in 1988. This represents > 75% of total pulp production of which more than half is bleached [2, 154]. Given the relatively low value of bleached kraft pulp, commercially viable processes will require massive quantities of inexpensive enzyme.

Recent trials with a variety of hemicellulases show substantial reductions in chlorine consumption. and paper properties (e.g. strength, brightness) are comparable to conventional chlorine bleaching [155–159]. The mechanism(s) involved is unclear, in part because chemical modifications of lignin and hemicellulose occurring during alkali cooking are poorly defined. However, it is clear that kraft pulps, particularly when derived from hardwoods, retain substantial concentrations of deacetylated xylan components. Further, a portion of the modified hemicellulose is redeposited onto cellulose fibers during the process [154]. It has been suggested that the hemicellulases cleave lignin-carbohydrate bonds or that swelling of the pulps facilitates extraction of residual lignin.

Future development of enzymatic bleaching processes will be dependent on availability of large quantities of inexpensive, highly specific enzymes. Even small amounts of contaminating cellulase activity drastically reduce paper strength, thus highly purified preparations have been sought [157–160]. Further improvements require consideration of the physical characteristics of the enzyme and their relationship to current kraft processes. In practical terms, this means that enzyme treatments will beat a high temperature, alkaline pH, and last only for a short duration. Pedersen *et al. [158]* have used alkaline xylanases for treating softwood kraft pulps. Several commercial enzyme preparations are currently available [161], and recombinant enzyme sources are under investigation [128, 131– 133, 162].

Ultimately. increased understanding of the mechanism(s) of enzymatic bleaching will facilitate process development. Recent studies have examined accessibility of the substrate to xylanases [163], the adsorption of xylanase to lignin and cellulose [164], and the presence of inhibitory materials in pulp [164].

Hemicellulases also show promise for the production of dissolving chemical pulps [165] and for improving pulp beatability of unbleached pulps [156, 166]. Complete removal of hemicellulose is sought in dissolving pulps to allow more efficient synthesis of cellulose derivatives (e.g. rayon) [154]. This is in distinction to papermaking where exhaustive removal of xylans reduces strength characteristics.

Recently, a combination of xylanase and peroxidase treatments has been

shown to be useful [167]. A multi-step process is described in which at least one xylanase treatment followed by one or more peroxidase treatments effectively delignifies pulps. Hardwood and softwood kraft pulps and either manganese peroxidase or lignin peroxidase could be used.

4.4 **Lignin degradation**

Unlike cellulose and hemicellulose, the lignin polymer is not principally linear nor does it have a repeating hydrolyzable interunit bond. Instead, lignin is a complex, three-dimensional, non-stereoregular aromatic polymer composed of phenylpropanoid units linked through several major types of carbon–carbon and ether bonds (Figure 4.3). It is synthesized by higher plants, reaching levels of 20–30% of the dry weight in the tissue of woody plants. The bulk of the lignin is in the thick secondary cell walls, but the highest concentrations of lignin are in the middle lamellae (intercellular regions) where the lignin cements the plant cells together thereby providing rigidity and strength for the plant.

The highly irregular structure of lignin is the result of free radical polymerization of the precursors, *p*-coumaryl, coniferyl, and sinapyl alcohols, the relative proportions depending on the type of plant and tissue. Plant

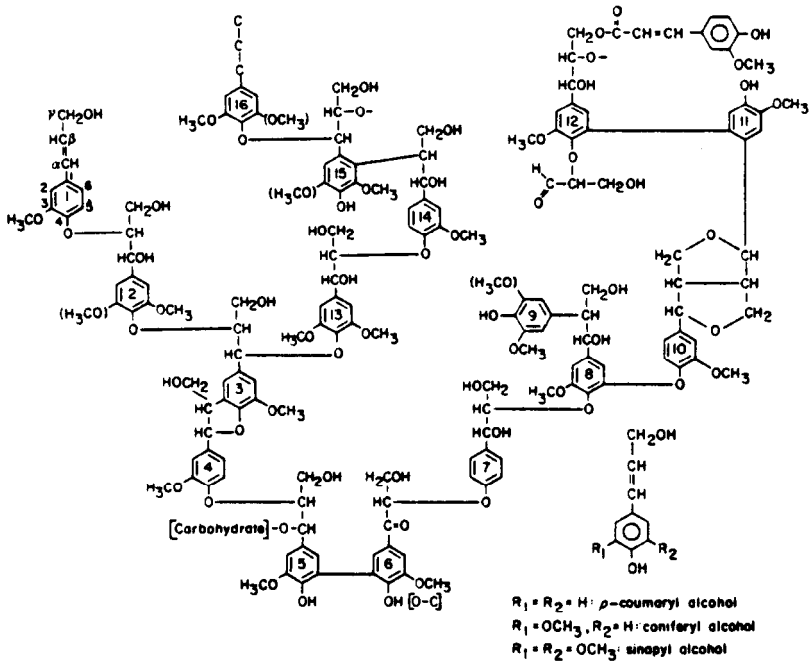

Figure 4.3 Structure of lignin and precursors. An example of a *β-O-4* linkage is shown between residues 1 and 2, and a *β-1* between residues 8 and 9. (From Kirk and Farrell [195].)

peroxidases catalyse the one-electron oxidation of these precursors to generate phenoxy radical intermediates that diffuse away from the enzyme and couple with each other and the growing lignin polymer. This random coupling generates oligomeric quinone methides susceptible to nucleophilic attack at the benzyl carbons by water, phenolic hydroxyls of other lignols. and also by the hydroxyls of hemiceilulose to form lignin-carbohydrate complexes. The interunit bonds are characterized by the points of attachment. The standard nomenclature designates positions on the propyl side chains as α, β, γ, (α being proximal to the aromatic ring) and positions on the aromatic ring as 1-6 (indicating the point of attachment of the propyl side chain). Thus. the β-*O*-4 ether bond is quantitatively the most important interunit bond in spruce lignin. Details of lignin synthesis, structure and chemistry are reviewed by Adler [168], Harkin [169], Higuchi [170], and Sarkanen and Ludwig [171].

The white-rot basidiomycetes degrade lignin more extensively and rapidly than any other known group of organisms. These fungi are also well adapted for utilizing the other major plant components. Because the complex lignin polymer encrusts the cellulosic microfibrils of plants and is chemically bonded to the hemicelluloses, the fungi capable of lignin degradation in fully lignified tissue play a key role in the recycling of carbon, not only from the lignin polymer, but also from the plant polysaccharides.

The most extensively characterized white-rot fungus is *P. chrysosporium*, previously known as *Chrysosporium lignorum* and *Sporotrichum pulverulentum [172, 173]*. In addition to *P. chrysosporium* being one of the fastest lignin degraders so far characterized, it also grows rapidly on defined glucose media. has a high temperature optimum for growth, conidiates profusely, and produces basidiospores under established conditions. making it particularly suited for study in the laboratory.

Important culture parameters required by *Phanerochaete* for lignin degradation to carbon dioxide and water have been determined using ^{14}C-1ignin as the substrate in defined media. Ligninolysis is triggered by nutrient limitation; cultures starved for carbon, nitrogen. or sulfur are able to oxidize lignin to carbon dioxide [174–177]. There is also great influence by oxygen partial pressure [176- 178], agitation [176, 179], metal ion balance [174] and pH [176]. To induce the ligninolytic system, the fungus is typically grown in stationary liquid medium at pH 4.5, 39°C, high oxygen partial pressure, with glucose as the carbon source and ammonium as the limiting nitrogen source. Although lignin is not required to induce the ligninolytic system. *P. chrysosporium* synthesizes veratryl alcohol (3,4-dimethoxybenzyl alcohol), which has a lignin aromatic substituent pattern [180,181].

A defined medium for lignin degradation has allowed detailed studies of the metabolism of lignin-model compounds (e.g., β-*O*-4 and β-1 lignin substructure models) without the complications associated with the complex heterogeneous lignin polymer. The oxidations of the lignin substructures (e.g. C_x-C_g

cleavage and C_x oxidation) [182– 189] agree well with those deduced from the studies with lignin polymer [190–192].

The ability of many other organisms to degrade lignin is somewhat uncertain. This is partially due to the inability of microbes, including the white-rot basidiomycetes, to use lignin as the sole source of carbon or energy. Consequently, there is no easy selection for lignin degradation by conventional methods. Also, the characterizations of minor modification and depletion of lignin in native lignocellulose are often equivocal due to its intimate association with the other plant constituents. However, other fungi such as certain ascomycetes and fungi imperfecti typify the soft-rot decay of wood. This is characterized by softening under high humidity conditions with extensive polysaccharide and lignin loss, but the rates are not as high as they typically are for the white-rot fungi. Other basidiomycetes, even closely related taxonomically to the white-rot fungi, cause brown rot. This is characterized by extensive decay of the hemicellulose and cellulose of wood, leaving a brown modified lignin residue [193]. The microbiology of lignin degradation has been reviewed [3, 194-196].

4.4.1 *Enzymology*

For the purposes of this chapter, the enzymology of lignin biodegradation will be limited principally to those enzymes secreted by *P. chrysosporium.* Growing evidence indicates that the lignin peroxidase (or ligninase) plays a central role in the initial degradation of the complex aromatic polymer lignin with this organism. Lignin peroxidase was first discovered based on the H_2O_2-dependent C_x-C_β cleavage of lignin model compounds and subsequently shown to catalyse the partial depolymerization of methylated lignin *in vitro* [197-200]. Since the first reports of lignin peroxidase. isozymic forms have been detected. The principal methods for distinguishing these have been by their *pI* and their order of elution from a Mono Q anion exchange column [201-203]. Ten peroxidases are separated by Mono Q chromatography and are designated H1 through to H10. Six of these, H1 *(pI* 4.7), H2 *(pI* 4.4), H6 *(pI* 3.7), H7 *(pI* 3.6), H8 *(pI* 3.5), and H10 *(pI* 3.3), have veratryl alcohol oxidation activity characteristic of lignin peroxidase [204]. H3, H4, H5, and H9 are manganese peroxidases (discussed below). Analytical isoelectric focusing has resolved fifteen proteins with lignin peroxidase activity [202]. The number of isozymes observed depends on the growth conditions (e.g. N v. C starved), the means of purification and storage. The isozymes are glycoproteins of molecular weights estimated at 38-46 kDa and also appear to be structurally related in that polyclonal antibodies raised against H8 cross react with the various other lignin peroxidases. It is clear from N-terminal and genetic analyses that some of these lignin peroxidases are separate gene products and not solely due to post-translational modifications (see below). Although the various lignin peroxidases have similar substrate specificities

towards aromatic substrates [204, 205], their kinetic behavior varies more clearly when *tert-butyl* hydroperoxide is substituted for hydrogen peroxide [205]. Crystallization of a lignin peroxidase should allow the determination of the three-dimensional structure and key structure/function relationships [206].

Lignin peroxidases are true peroxidases [207-209] and the kinetics of enzyme intermediates have been studied in detail [210–212]. Lignin peroxidase isozymes have absorption spectra characteristic of other hemeproteins, and pyridine hemochromogen complex formation indicates 1 mol of protoheme IX per mole of lignin peroxidase. Like other peroxidases, lignin peroxidase has a ping-pong mechanism, i.e. hydrogen peroxide oxidizes resting enzyme by two electrons to give Compound I enzyme intermediate. Compound I oxidizes aromatic substrates by one electron to give Compound II (a one-electron oxidized enzyme intermediate) that can again oxidize substrate to return the enzyme to resting state. This is depicted in the following in which LiP represents the ferric state resting lignin peroxidase and S represents an aromatic substrate

$$\text{LiP} + \text{H}_2\text{O}_2 \rightarrow \text{Compound I} + \text{H}_2\text{O}$$

$$\text{Compound I} + \text{S} \rightarrow \text{Compound II} + \text{S}^{+\bullet}$$

$$\text{Compound II} + \text{S} \rightarrow \text{LiP} + \text{S}^{+\bullet}$$

Lignin peroxidase catalyses a variety of oxidations, all of which are dependent on H_2O_2. These include C_α–C_β cleavage of the propyl side chains of lignin and lignin models, hydroxylation of benzylic methylene groups, oxidation of benzyl alcohols to the corresponding aldehydes or ketones, phenol oxidation, and even aromatic ring cleavage of non-phenolic lignin model compounds [200, 203, 213–215]. The underlying principle behind this array of reactions was initially perplexing but can now be explained. Simply stated, lignin peroxidase oxidizes the aromatic nuclei of substrates by one electron (Figure 4.4). The resulting aryl cation radicals degrade spontaneously via many reactions dependent on the structure of the substrate and on the presence of reactants (Figure 4.5). Evidence for the involvement of cation radical intermediates was provided using electron spin resonance [216], using

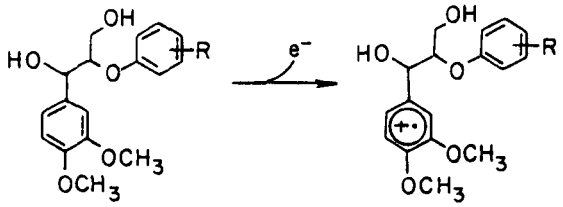

Figure 4.4 Formation of aryl cation radical intermediate by the oxidation of substrate by one electron.

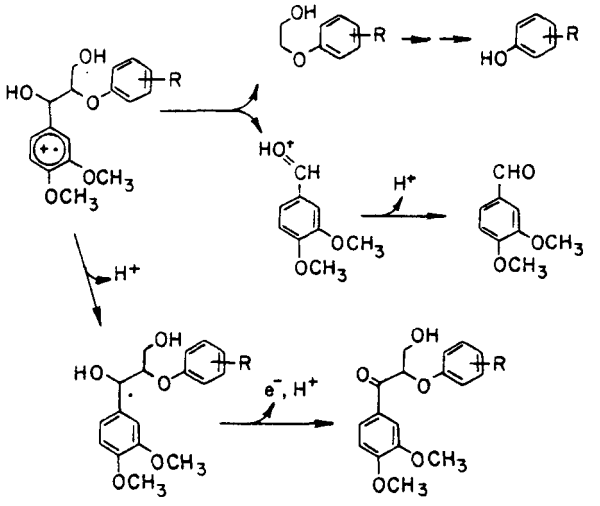

Figure 4.5 C_α–C_β cleavage and C_α oxidation as a result of spontaneous reactions subsequent to aryl cation radical formation. (Adapted from Kirk and colleagues [220].)

purified lignin peroxidase and methoxybenzenes as substrates. Using more lignin-related compounds. Hammel *et al.* [217] further proved the involvement of radical intermediates by identifying radical-dimer products as well as carbon-centered and peroxyl radical intermediates. Other researchers, using more indirect methods, also concluded that cation radical intermediates were involved [218, 219].

Although the reactions subsequent to cation radical formation are complex. they follow chemical principles that readily explain the predominant products. Thus, the C_α–C_β cleavage (Figure 4.5) that is predominant with model compounds most closely related to lignin [217, 220] can be understood based on the degree of substitution of the C_β carbon stabilizing a C-centered radical in purely chemical systems [221]. The cation radical mechanism also explains the oxygenase properties of lignin peroxidase; O_2 reacts with carbon-centered radical intermediates that result from C-C bond cleavage of cation radicals [222, 223]. Lignin peroxidase activity is also expected to break the C_α lignin-carbohydrate bond [220]. Detailed reviews on the radical chemistry of lignin peroxidase catalysed reactions are provided elsewhere [170, 224].

A distinguishing characteristic of lignin peroxidase is that its oxidized enzyme intermediates (Compound I and Compound II) must be sufficiently positive to remove one electron from its non-phenolic substrates. Hammel *et al.* [225] demonstrated that lignin peroxidase oxidizes polycyclic aromatics with ionizing potentials of approximately 7.5 eV or lower, whereas horseradish peroxidase will oxidize only those polycyclics with ionization potentials below 7.35 eV [226]. Similarly, the homologous series of methoxybenzenes (half-wave potentials range from 0.81 V to 1.76 V v. a saturated calomel electrode) has been useful in characterizing the relative oxidation potentials of

oxidative enzymes. in the presence of H_2O_2, lignin peroxidase oxidizes the ten congeners with the lowest half-wave potentials. whereas horseradish peroxidase oxidizes the four lowest and laccase (with O_2 as electron acceptor) oxidizes only the lowest [227]. The higher E_{m7} values of the ferrous–ferric states with lignin peroxidases. when compared to other peroxidases, also suggest that the heme active site of lignin peroxidase is more electron deficient, thereby allowing the oxidized intermediates of the enzyme to oxidize substrates of high oxidation-reduction potential [228].

Consistent with the characterization of lignin peroxidase as a peroxidase are the polymerization reactions it catalyses with phenols. This is of special interest because phenolics are produced by the action of lignin peroxidase on non-phenolic lignin structures. and therefore explains why the treatment of lignin with the enzyme can cause not only the depolymerization of lignin [199] but also the repolymerization of lignin fragments *in vitro* [67, 229]. Recently, the partial depolymerization of a synthetic lignin with crude lignin peroxidase and H_2O has been demonstrated *in vitro* [230]. Dilute lignin dispersions and low steady-state H_2O_2 concentrations are thought to be important in minimizing the bimolecular coupling of phenoxy radicals that would lead to polymerization *in vitro* [230]. It has also been proposed that glycosylation of lignin breakdown products may be important in favoring the depolymerizing reactions [231].

The importance of lignin peroxidase in depolymerization of lignin *in vivo* was convincingly demonstrated by Leisola *et al.* [232]. Addition of exogenous lignin peroxidase to carefully washed mycelial pellets greatly stimulated the conversion of ^{14}C-lignin to $^{14}CO_2$. In this case, the presence of mycelia may play an important role in favoring overall depolymerization by removing lignin fragments as they are released. Adding veratryl alcohol. in addition to the lignin peroxidase, to the washed mycelia had a further stimulatory effect. Conversely, horseradish peroxidase had no effect.

Another heme peroxidase found in the extracellular fluid of ligninolytic cultures of *P. chrysosporium* is manganese peroxidase [233, 234]. The principal function of the enzyme is to oxidize Mn^{2+} to Mn^{3+} using H_2O_2 as oxidant. Activity of the enzyme is stimulated by simple organic acids that stabilize the Mn^{3+} and allow it to oxidize organic compounds including phenolic lignin model compounds [235, 236]. As with lignin peroxidase, the prosthetic group is iron protoporphyrin IX and several isozymes can be detected [202, 237–239]. The 46 kDa glycoprotein does not share antigenic determinants with lignin peroxidase, and peptide patterns are different to those observed with lignin peroxidase [202].

Manganese peroxidase exhibits enzyme intermediates analagous to other peroxidases [239, 240]. Compound I is formed by the oxidation of manganese peroxidase by H_2O_2, and this enzyme intermediate can then be reduced by Mn^{2+} and phenols to generate Compound II. Compound II is then reduced back to resting state by Mn^{2+} but not by phenols [240]. Therefore, Mn^{2+} is

necessary to complete the catalytic cycle. The enzyme also shows saturation kinetics with Mn^{2+}

Typical assays for monitoring manganese peroxidase activity use phenolics that are easily oxidized by Mn^{3+} and that give useful color changes for detection. However, there has been some question as to the role of the enzyme, in particular in relation to oxidation of lignin and its depolymerization. The biomimetic oxidation of lignin model compounds by Mn^{3+} suggests that it may play a role in oxidizing both phenolic and non-phenolic residues of lignin [241]. More recently, the *in vitro* partial depolymerization of synthetic lignin by manganese peroxidase has been demonstrated [242].

Clearly, an important component of the ligninolytic system of *P. chrysosporium.* in addition to the extracellular peroxidases, is the source of H_2O_2 required as oxidant. Several oxidases have been proposed to play a role in this regard. However, the only one that appears to be secreted in this system under standard ligninolytic conditions is glyoxal oxidase [195].

The temporal correlation of glyoxal oxidase. peroxidase, and oxidase substrate appearances in cultures suggests a close physiological connection between these components [243, 244] The oxidase is a glycoprotein of 68 kDa with two isozymic forms *(pI* 4.7 and 4.9). The active site of the enzyme has not been characterized but Cu^{2+} appears to be important in maintaining activity of purified enzyme. Glyoxal oxidase is produced in cultures when *P. chrysosporium* is grown on glucose or xylose, the major sugar components of lignocellulosics. However, the physiological substrates for glyoxal oxidase are not these growth-carbon compounds but apparently intermediary metabolites. Several simple aldehyde. α-hydroxycarbonyl, and α-dicarbonyl compounds are substrates. Interestingly, proposed products of ligninolysis. such as glycoladehyde [200], are also substrates for glyoxal oxidase. suggesting that the oxidase-peroxidase coupled system can partially be perpetuated by the action of lignin peroxidase on lignin itself [244].

Perhaps the property of glyoxal oxidase of most interest and of considerable physiological significance is that, in the absence of a peroxidase system. the oxidase is reversibly inactivated [244]. However. the enzyme is reactivated by reconstituting the complete peroxidase system. including both lignin peroxidase and substrate. This suggests that the supply of H_2O_2 by glyoxal oxidase is responsive to the demand of the peroxidases, thereby providing an extracellular regulatory mechanism for control of the coupled enzyme systems. On a protein basis, the amount of glyoxal oxidase appears to be relatively minor when compared to the extracellular peroxidases. However. the specific activity of the oxidase appears to be considerably greater than that of lignin peroxidase. Interestingly, the relative amounts of glyoxal oxidase appear to be much greater when extracted from wood solid substrate compared to defined liquid medium [245].

Another oxidase secreted by *P. chrysosporium* is cellobiose oxidase previously described. This enzyme is not normally detected in standard ligninolytic

cultures in the laboratory, perhaps as a result of repression by glucose in cultures. However, the possibility that it may play a role in supplying H_2O_2 during lignin degradation on native lignocellulosic substrates has not been excluded. Also, an extracellular aryl-alcohol has been reported from the white-rot fungus *Bjerkandera adusta* as well as a lignin-peroxidase [246].

Unlike many white-rot fungi, *Phanerochaete* has no detectable laccase activity. However, recent evidence suggests that laccase. like lignin peroxidase, plays a role in lignin degradation by fungi [247-249]. The laccase of *Trametes versicolor is* a blue copper oxidase that catalyses the four-electron reduction of O_2 to H_2O during its oxidation of phenolics, aromatic amines, ascorbate, and metal cyanides [250]. The enzyme has four copper atoms with type-1 and type-3 copper sites of particularly high redox potential in comparison to other blue copper proteins [251]. As with lignin peroxidase, the substrate radicals formed subsequent to oxidation undergo further nonenzymatic reactions [252]. The chemistry of laccase oxidation of lignin-related compounds has recently been reviewed [253]. Also, the synergism between the laccase and manganese peroxidase of *Rigidoporus lignosus* is an example of the possible complex relationships that may be important in lignocellulose degradation [254].

4.4.2 *Molecular genetics of ligninolytic fungi*

Knowledge concerning the molecular genetics of the *P. chrysosporium* ligninolytic system has advanced considerably in the past few years. Standard procedures have been established for auxotroph production [255], recombination analysis [74], and rapid DNA and RNA purification [256, 257].

Several groups have developed transformation systems for *P. chrysosporium.* Randall and co-workers [258] reported transformation to G418 resistance. This system appears to involve extremely low copy numbers of autonomously replicating plasmid. An *adel* strain of *P. chrysosporium* has been transformed with the corresponding wild-type gene from *Schizophyllum commune* [80, 81], and from *P. chrysosporium [259].* These transformants involved stable integration of the complementing vectors into the genome, although the frequency of integration at the homologous *adel* locus has not been determined. Homologous recombination frequencies are high in *Ustilago maydis* [260] and relatively low in *Coprinus cinereus* [261], the only basidiomycetes where this has been examined and reported. This issue is important because it dictates strategies required for gene disruptions/ replacements.

Since Tien and Tu [262] first reported cloning of the cDNA encoding lignin peroxidase H8, much has been learned concerning the number, structure, and organization of the peroxidase genes of *P. chrysosporium.* It is now clear that lignin peroxidases are encoded by a family of at least six closely related genes and their allelic variants, and that many of these genes are linked. Regrettably,

Table 4.1 Nucleotide homologies based on optimized alignments of nucleotides. −100 bp of translational start codons to +100 bp beyond translational stops are shown in parentheses following coding sequence homology. Only cDNA sequence is available for CLG4 (266).

	Amino acid							
	H8	LiPA	O282	LPOB	LiPB	GLG5	GLG2	CLG4
H8		99.5	96.5	91.1	91.4	82.6	82.8	72.1
LiPA	99.0(97.8)		96.5	91.4	91.9	82.6	82.6	71.8
O282	88.1(79.8)	87.5(79.9)		89.5	89.8	81.2	81.2	70.5
LPOB	86.6(73.6)	86.2(76.6)	84.3(72.8)		99.5	81.0	81.0	69.7
LiPB	86.9(76.1)	86.7(76.3)	84.4(72.3)	98.5(96.3)		81.2	81.2	69.4
GLG5	80.8(70.5)	80.9(70.4)	78.4(70.2)	79.3(69.0)	79.9(71.4)		100	66.8
GLG2	80.5(70.5)	80.6(70.0)	78.1(69.2)	79.3(69.0)	79.9(70.4)	99.2(97.5)		66.8
CLG4	73.7	73.2	71.1	73.3	73.1	71.8	72.1	

Nucleotide

no uniform nomenclature has been adopted for clone/gene designations. Also, when some sequences were initially published, their allelic relationships had not been established. A list of BKM- 1767 synonyms follows:

1. ML1, cDNA encoding isozyme H8 [262]
2. ML4, cDNA encoding allelic variant of ML1 [263]
3. LPOA, genomic clone corresponding to ML1 [264, 265]
4. LiPA, genomic clone corresponding to ML4 [76]
5. CLG4, a cDNA [266]
6. CLG5, a cDNA [266]
7. GLG5, genomic clone corresponding to CLG5 [76]
8. GLG2, genomic clone probably allelic to CLG5/GLG5 [267]
9. O282, genomic clone [75]
10. LiPB, genomic clone allelic to LPOB [76, 265]
11. V4, genomic clone partially sequenced [75].

Sequence, intron position, and intron length are highly conserved among lignin peroxidase genes. Nucleotide and amino acid sequence homology among clones derived from *P. chrysosporium* BKM-1767 are shown in Table 4.1. The lignin peroxidase genes contain eight relatively small introns, except GLG5 contains nine [76]. Intron position is conserved particularly around residues Arg-43, His-47, and His-176, which are believed to be essential for peroxidase activity [262] (Figure 4.6). Also, the last intron of all clones is adjacent to a short, proline-rich exon, and the first intron splits a putative signal sequence-propeptide junction in several genes [75].

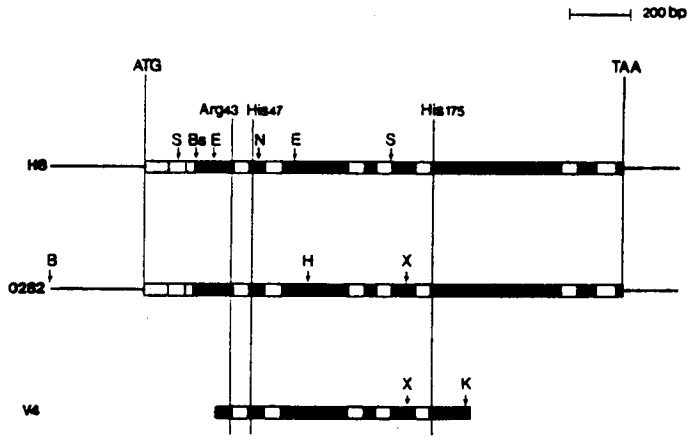

Figure 4.6 Representation of genomic clone showing alignments of signal sequences (stippled box). Introns (open box), and coding regions for mature polypeptide (blackened box). The positions of essential amino acid residues are shown by cDNA coordinates [262]. For cosmid V4, only sequences surrounding essential amino acid residues were determined. Restriction site abbreviations: S, SphI; Bs, BssHII; E, EcoRV; N, NcoI; H, HindIII; X, *XhoI;* K. KpnI.

A cDNA encoding manganese peroxidase [268] and its corresponding genomic clone [269] have been isolated from a derivative of strain ME446. A manganese peroxidase cDNA sequence derived from BKM - 1767 has also been reported [270]. Amino sequence homology to lignin peroxidase H8 is 50–60%, and the residues essential for peroxidase activity are conserved.

The lignin peroxidase genes are linked. An RFLP-based genetic map localized lignin peroxidase genes of M E446 to two linkage groups [77]. Following chromosome separation by clamped homogeneous electrical field (CHEF) electrophoresis, five lignin peroxidase genes have been assigned to a single chromosome [76]. In agreement with the RFLP map, another lignin peroxidase clone (GLG4) has been assigned to the same chromosome as a *cbh1* cluster [104]. The number of manganese peroxidase genes and their chromosomal organization have not been reported. although preliminary (unpublished) results show that at least one manganese peroxidase gene resides on the same chromosome as live lignin peroxidase genes.

Both lignin peroxidase-containing chromosomes are dimorphic with respect to migration on CHEF gels [76]. Homologous chromosomes differing in electrophoretic mobility have also been observed in *Plasmodium falciparurn [271], S. cerevisiae [272], Candida albicans [273], U. maydis [274],* and C. *cinereus* (M. Zolan, personal communication). The mechanism(s) giving rise to these chromosome-length polymorphisms may be related to variations in lignin peroxidase activity. In *P. falicurum,* polymorphisms are caused by homologous recombination in distal regions of chromosomes. and it has been suggested that recombination in these dynamic regions may be related to antigenic variation [271]. Extensive mapping of chromosome homologies will be required to determine if a similar mechanism is operative in *P. chrysosporium.*

Studies of the transcriptional regulation of peroxidases have been hampered by difficulties in distinguishing closely related genes. Although it is clear that lignin peroxidase genes are transcriptionally regulated [262, 263, 275] and that expression of manganese peroxidase genes is Mn-dependent [276], the specificity of the transcripts observed on Northerns is questionable. S1 mapped transcriptional start points are equally tenuous [275, 277].

In addition to confusion concerning transcript specificity, considerable uncertainty remains concerning the precise translational products of various clones. Identification by matching deduced amino acid sequence with short regions of experimentally determined sequence is particularly risky [266]. This is also complicated by the presence of allelic forms that encode slightly different isozymes [76]. Recently, Glumoff *et al.* [205] purified five lignin peroxidase isozymes from carbon-starved cultures and determined their N-terminal amino acid sequences. On this basis, three isozymes with *pIs* of 4.65, 3.85, and 3.70 can be tentatively identified as products of CLG4, LiPA/LPOA, and LiPB/LPOB, respectively.

Large quantities of purified lignin peroxidases are needed for basic

biochemical investigations and for assessing various commercial applications. Unfortunately, expression of lignin peroxidase genes in heterologous systems has proven to be problematic. In *E. coli,* relatively low levels of aggregated apoprotein are produced in inclusion bodies. Attempts to recover and reconstitute the active enzyme have had limited success (reviewed in [278]). *S. cerevisiae* systems are also inadequate, yielding little or no intracellular levels of apoprotein [278]. Even when under the control of the highly expressed and inducible *cbhl* promoter, expression of peroxidases in *T. reesei is* limited to transcripts only, i.e., no protein is detected [279]. Recently, a baculovirus expression system has been used to produce active recombinant lignin peroxidase isozyme H8 [280]. Although not appropriate for large-scale production, such systems may be useful for experiments requiring limited quantities of recombinant protein, e.g. site specific mutagenesis.

Little is known concerning the molecular genetics of other lignin degrading fungi. Saloheimo and co-workers [279] cloned and sequenced a lignin peroxidase gene from *Phlebia radiata.* The deduced amino acid sequence of this clone is 62%, identical to *P. chrysosporium* isozyme H8 (ML1). On the basis of Southern blot hybridization to the H8 gene. multiple lignin peroxidases appear to be present in *Bjerkandera adjusta. Coriolus versicolor* and *Fomes lignosus [265].* A phenol oxidase gene of *Colletotrichurn hirsutus* has been cloned, sequenced and expressed in *S. cerevisiae [281].*

4.5 Applications

A potential application for the ligninolytic system of white-rot fungi is the biomechanical pulping process previously described. The mechanism of biopulping is not well understood but a working hypothesis is that the limited removal of lignin facilitates the separation of the cellulose microfibrils. Elucidation of the mechanism has been hampered by the complexity of the substrates, although lignin peroxidase. manganese peroxidase, and glyoxal oxidase have been identified in *P. chrysosporium* cultured on aspen pulp [245].

The non-specific oxidative ligninolytic system of white-rot fungi also has potential in solving certain environmental problems. The first alkaline extraction stage of Kraft pulp bleach plants contains oxidized and chlorinated lignin fragments and a complex mixture of low molecular aromatics that area major source of water pollution. *P. chrysosporium* can decolonize the effluent, degrade the lignin residues, and remove the chloro-aromatics [282-284].

P. chrysosporium also is able to oxidize and. in some cases, mineralize to CO_2 and H_2O certain xenobiotics that are not lignin derived. Examples are polychlorinated biphenyls [285], benzo(a)pyrene, DDT, lindane [286], and other chloro-organics [284]. Recently, Lamar and co-workers have demonstrated depletion of the wood preservative pentachlorophenol in soils by *Phanerochaete* spp. [287]. The metabolism of these compounds appears to

correlate with the ligninolytic system, and in some cases the direct involvement of lignin peroxidase in oxidizing these compounds has been shown [225, 288–291]. Although lignin peroxidase may catalyze the initial oxidations of such xenobiotics, the subsequent steps are not well understood and should be an important goal of future research.

References

1. Meier, H. (1985) *Biosynthesis and Biodegradation of Wood Components* (Higuchi, T. ed.). Academic Press, New York, pp. 43–50.
2. Smook, G. (1982) *Handbook for Pulp and Paper Technologists*, Joint Textbook Committee of the Paper Industry, TAPPI, Atlanta, Georgia.
3. Eriksson, K.-E.L., Blanchette, R.A. and Ander, P. (1990) *Microbial and Enzymatic Degradation of Wood and Wood Components*, Springer-Verlag, Berlin.
4. Wood, T.M. and Garcia-Campayo, V. (1990) *Biodegradation* **1**: 147–161.
5. Beguin, P. (1990) *Ann. Rev. Microbiol.* **44**: 219–248.
6. Eriksson, K.E. and Wood, T.M. (1985) *Biosynthesis and Biodegradation of Wood Components* (Higuchi, T. ed.) Academic Press, New York, 469–503.
7. Coughlan, M.P. and Ljugdahl, L.G. (1988) *Biochemistry and Genetics of Cellulose Degradation*. (Aubert, J.P., Beguin, P. and Miller, J. eds). Academic Press, London, 11–30.
8. Knowles, J., Lethovaara, P. and Teeri, T. (1987) *TIBTECH* **5**: 255–261.
9. Eriksson, K.-E. (1978) *Biotechnol. Bioeng.* **20**: 317–332.
10. Wood, T.M. (1989) *Enzyme Systems for Lignocellulose Degradation* (Coughlan, M.P. ed.). Elsevier Applied Science, New York, 17–35.
11. Eriksson, K.-E. and Pettersson, B. (1975) *Eur. J. Biochem.* **51**: 193–206.
12. Eriksson, K.-E. and Pettersson, B. (1975) *Eur. J. Biochem.* **51**: 213–218.
13. Despande, V., Eriksson, K.-E. and Pettersson, B. (1978) *Eur. J. Biochem.* **90**: 191–198.
14. Sternberg, D. (1976) *Appl. Environ. Microbiol.* **31**: 648–654.
15. Vallander, L. and Eriksson, K.-E. (1987) *Enzyme Microb. Technol* **9**: 714–720.
16. Coughlan, M.P. (1985) *Biotechnol. Gen. Eng. Rev.* **3**: 39–109.
17. Henrissat, B., Driquez, H., Viet, C. and Schulein, M. (1985) *Bio/Technology* **3**: 722–726.
18. Fagerstam, L.G. and Pettersson, L.G. (1980) *FEBS Lett.* **119**: 97–100.
19. Wood, T.M. and McCrae, S.I. (1986) *Biochem. J.* **234**: 93–99.
20. Knowles, J.K.C., Lehtovaara, P., Murray, M. and Sinnott, M. (1988) *J. Chem. Soc., Chem. Commun.* 1401–1402.
21. Claeyssens, M., Tomme, P., Boewer, C.F. and Hehre, E.J. (1990) *FEBS Lett.* **263**: 89–92.
22. Niku-Paavola, M.L., Lappalainene, A., Enari, T.M. and Nummi, M. (1985) *Biochem. J.* **231**: 75–81.
23. Chanzy, H., Henrissat, B. and Vuong, R. (1984) *FEBS Lett.* **172**: 193–197.
24. Enari, T.M. and Niku-Paavola, M.L. (1987) *CRC Crit. Revs. Biotechnol.* **5**: 67–87.
25. Wood, T.M., McCrae, S.I. and Bhat, K.M. (1989) *Biochem. J.* **260**: 37–43.
26. Van Tilbeurgh, H., Bhikhabhai, R., Pettersson, L.G. and Claeyssens, M. (1984) *FEBS Lett.* **169**: 215–218.
27. Knowles, J.K., Teeri, T.T., Lehtovaara, P., Penttila, M. and Saloheimo, M. (1988) *Biochemistry and Genetics of Cellulose Degradation*. (Aubert, J.P., Beguin, P. and Miller, J. eds). Academic Press, London, 153–169.
28. Highley, T. and Wolter, K.E. (1982) *Mater. Org.* **17**: 127–134.
29. Highley, T.L. (1973) *Wood Fiber*, **5**: 50–58.
30. Illman, B. and Highley, T. (1989) *Biodeterioration Research II*. (O'Rear, C.E. and Llewellyn, G.E. eds). Plenum Press, New York, 465–484.
31. Shoemaker, S., Schweickart, V., Ladner, M., Gelfand, D., Kwok, S., Myambo, K. and Innis, M. (1983) *Bio/Technology* **1**: 691–696.
32. Teeri, T., Salovuori, I. and Knowles, J.K. (1983) *Bio/Technology* **1**: 696–699.
33. Chen, C.M., Gritzali, M. and Stafford, D.W. (1987) *Bio/Technology* **5**: 274–278.

34. Teeri, T., Lehtovaara, P., Kauppinen, S., Salovuori, I. and Knowles, J. (1987) *Gene* **51**: 43–52.
35. Penttila, M., Lehtovaara, P., Nevalainen, H., Bhikabhai, R. and Knowles, J. (1986) *Gene* **45**: 253–263.
36. Saloheimo, M., Lehtovaara, P., Penttila, M., Teeri, T., Stahlberg, J., Johansson, G., Claeyssens, M., Tomme, P. and Knowles, J.K. (1988) *Gene* **63**: 11–21.
37. Barnett, C., Berka, R. and Fowler, T. (1991) *Bio/Technology* **9**: 562–567.
38. Sims, P., James, C. and Broda, P. (1988) *Gene* **74**: 411–422.
39. Azevedo, M., Felipe, M.S., Astolfi-Filho, S. and Radford, A. (1990) *J. Gen. Microbiol.* **136**: 2569–2576.
40. Penttila, M., Nevalainen, H., Raynal, A. and Knowles, J.K. (1984) *Mol. Gen. Genet.* **194**: 494–499.
41. Claeyssens, M. and Tomme, P. (1989) *Enzyme Systems for Lignocellulose Degradation*, (Coughlan, M.P. ed.). Elsevier Applied Science, New York 37–49.
42. Van Tilbeurgh, H., Tomme, P., Claeyssens, M., Bhikhabhai, R. and Pettersson, G. (1986) *FEBS Lett.* **204**: 223–227.
43. Stahlberg, J., Johansson, G. and Pettersson, G. (1991) *Bio/Technology* **9**: 286–290.
44. Tomme, P., van Tilbeurgh H., Pettersson, G., van Damme, J., Vandekerckhove, J., Knowles, J., Teeri, T. and Claeyssens, M. (1988) *Eur. J. Biochem.* **170**: 575–581.
45. Tomme, P., Heriban, V. and Claeyssens, M. (1990) *Biotechnol. Lett.* **12**: 525–530.
46. Kraulis, P.J., Clore, G.M., Nilges, M., Jones, T.A., Pettersson, G., Knowles, J.K. and Gronenborn, A.M. (1989) *Biochemistry* **28**: 7241–7257.
47. Abuja, P.M., Pilz, I., Claeyssens, M. and Tomme, P. (1988) *Biochem. Biophys. Res. Commun.* **156**: 180–185.
48. Abuja, P.M., Schmuck, M., Pilz, I., Tomme, P., Claeyssens, M. and Esterbauer, H. (1988) *Eur. Biophys. J.* **15**: 339–342.
49. Stahlberg, J., Johansson, G. and Pettersson, G. (1988) *Eur. J. Biochem.* **173**: 179–183.
50. Gilkes, N.R., Warren, R., Miller, R. and Kiburn, D. (1988) *J. Biol. Chem.* **263**: 10401–10407.
51. Paice, M.G., Desrochers, D., Rho, D., Jurasek, J., Roy, C., Rollin, C.F., Migel, E.D. and Myaguchi, M. (1984) *Bio/Technology* **2**: 535–539.
52. Johansson, G., Stahlberg, J., Lindeberg, G., Engstrom, A. and Pettersson, G. (1989) *FEBS Lett.* **243**: 389–393.
53. Bergfors, T., Rouvinen, J., Lethovaara, P., Caldentey, X., Tomme, P., Claeyssens, M., Pettersson, G., Teeri, T., Knowles, J. and Jones, T. (1989) *J. Mol. Biol.* **209**: 167–169.
54. Rouvinen, J., Bergfors, T., Teeri, T., Knowles, J.K.C. and Jones, T.A. (1990) *Science* **249**: 380–386.
55. Claeyssens, M., van Tilbeurgh, H., Tomme, P., Wood, T.M. and McCrae, S.I. (1989) *Biochem. J.* **261**: 819–825.
56. Henrissat, B., Claeyssens, M., Tomme, P., Lemesle, L. and Mornon, J.P. (1989) *Gene* **81**: 83–95.
57. Tomme, P. and Claeyssens, M. (1989) *FEBS Lett.* **243**: 239–343.
58. Clarke, A.J. and Yaguchi, M. (1985) *Eur. J. Biochem.* **149**: 233–238.
59. Yaguchi, M., Roy, C., Rollin, C.F., Paice, M. and Jurasek, L. (1983) *Biochem. Biophys. Res. Commun.* **116**: 408–411.
60. Eriksson, K.-E. and Hamp, S.G. (1978) *Eur. J. Biochem.* **90**: 183–191.
61. Messner, R. and Kubicek, C.P. (1991) *Appl. Environ. Microbiol.* **57**: 630–635.
62. Mandels, M. and Reese, E.T. (1960) *J. Bacteriol.* **79**: 816–826.
63. El-Gogary, S., Leite, A., Crivellaro, O., Eveleigh, D.E. and El-Dorry, H. (1989) *Proc. Natl. Acad. Sci. USA* **86**: 6138–6141.
64. Fritscher, C., Messner, R. and Kubicek, C.P. (1990) *Exp. Mycol.* **14**: 405–415.
65. Ayers, A.R., Ayers, S.B. and Eriksson, K. (1978) *Eur. J. Biochem.* **90**: 171–181.
66. Westermark, U. and Eriksson, K.-E. (1974) *Acta Chem. Scand.* **28**: 209–214.
67. Odier, E., Mozuch, M.D., Kalyanaraman, B. and Kirk, T.K. (1988) *Biochemie* **70**: 847–852.
68. Bruchmann, E., Schach, H. and Graf, H. (1987) *Biotechnol. Appl. Biochem.* **9**: 146–159.
69. Renganathan, V., Usha, S.N. and Lindenburg, F. (1990) *Appl. Microbiol. Biotechnol.* **32**: 609–613.
70. Henriksson, G., Pettersson, G., Johansson, G., Ruiz, A. and Uzcategui, E. (1991) *Eur. J. Biochem.* **196**: 101–106.
71. Eriksson, K. and Pettersson, B. (1982) *Eur. J. Biochem.* **124**: 635–642.

72. Thompson, W. and Broda, P. (1987) *Tr. Br. Mycol. Soc.* **3**: 285–294.
73. Alic, M., Letzring, C. and Gold, M.H. (1987) *Appl. Environ. Microbiol.* **53**: 1464–1469.
74. Alic, M. and Gold, M.H. (1985) *Appl. Env. Microbiol.* **50**: 27–30.
75. Schalch, H., Gaskell, J., Smith, T.L. and Cullen, D. (1989) *Mol. Cell. Biol.* **9**: 2743–2747.
76. Gaskell, J., Dieperink, E. and Cullen, D. (1991) *Nuc. Acids Res.* **19**: 599–603.
77. Raeder, U., Thompson, W. and Broda, P. (1989) *Mol. Microbiol* **3**: 911–918.
78. Raeder, U., Thompson, W. and Broda, P. (1989) *Mol. Microbiol* **3**: 919–924.
79. Penttila, M., Nevalainen, H., Ratto, M., Salminen, E. and Knowles, J. (1987) *Gene* **61**: 155–164.
80. Alic, M., Kornegay, J., Pribnow, D. and Gold, M.H. (1989) *Appl. Environ. Microbiol.* **55**: 406–411.
81. Alic, M., Clark, E.K., Kornegay, J.R. and Gold, M.H. (1990) *Curr. Genet.* **17**: 305–312.
82. Munoz-Rivaz, A., Specht, C.A., Drummond, B.J., Froeliger, E., Novotony, C.P. and Ullrich, R.C. (1986) *Mol. Gen. Genet.* **205**: 103–106.
83. Gelfand, D., Innis, M., Kwok, S., Ladner, M. and Schweickart, V. (1985) Recombinant fungal cellobiohydrolases, European Patent Application No. 84110305.4.
84. Knowles, J., Penttila, M., Teeri, T., Nevalainen, H., Salovuori, I. and Lehtovaara-Helenius, P. (1985) Yeast strains producing cellulolytic enzymes and methods and means for constructing them, International Patent Publication No. WO 85/04672.
85. Van Arsdell, J., Kwok, S., Schweickart, V., Ladner, M., Gelfand, D. and Innis, M. (1987) *Bio/Technology* **5**: 60–64.
86. Penttila, M., Lehtovaara, P. and Knowles, J. (1989) *Yeast Genetic Engineering*, Barr, P., Brake, A. and Valenzuela, P. (eds.). Butterworth, Boston 247–267.
87. Nevalainen, H., Penttila, M., Harkki, A., Teeri, T. and Knowles, J. (1990) *Molecular Industrial Mycology: Systems and Applications*, (Leong, S.A. and Berka, R. eds). Marcel Dekker, New York.
88. Ong, E., Gilkes, N.R., Warren, R.A., Miller, R. and Kilburn, D.G. (1989) *Bio/Technology* **7**: 604–607.
89. Ong, E. (1991) *Enzyme Microb. Technol.* **13**: 59–65.
90. Greenwood, J.M., Gilkes, N.R., Kilburn, D.G., Miller, R.C. and Warren, R.A. (1989) *FEBS Lett.* **244**: 127–131.
91. Kirk, T.K. and Moore, W.E. (1972) *Wood and Fiber* **4**: 72–79.
92. Akamatsu, I., Yoshihara, H., Kamishima, H. and Fujii, T. (1984) *Mokuzai Gakk.* **30**: 697–702.
93. Samuelsson, L., Mjoberg, P.J., Hartler, L., Vallander, L. and Eriksson, K. (1980) *Svensk Papperstidn.* **83**: 221–225.
94. Eriksson, K. and Vallander, L. (1982) *Svensk Papperstidn.* **6**: 33–38.
95. Pilon, L., Varbe, M.C., Desrochers, M. and Jurasek, L. (1982) *Biotechnol. Bioeng.* **24**: 2063–2076.
96. Bar-Lev, S.S., Kirk, T.K. and Chang, H.M. (1982) *TAPPI J.* **65**: 111–113.
97. Leatham, G. and Myers, G. (1990) *TAPPI J.* **73**: 192–197.
98. Leatham, G., Myers, G. and Wegner, T. (1990) *TAPPI J.* **73**: 249–255.
99. Leatham, G., Myers, G. and Wegner, T. (1990) *TAPPI J.* **73**: 197–200.
100. Sachs, I., Leatham, G. and Myers, G. (1989) *Wood and Fiber Sci.* **21**: 331–342.
101. Eriksson, K., Jonsrud, S.C. and Vallander, L. (1983) *Arch. Microbiol.* **135**: 161–168.
102. Ruel, K., Joseleau, J., Jonsrud, S.C. and Eriksson, K. (1986) *Holzforschung* **40**: 5–9.
103. Eriksson, E.K. and Kirk, T.K. (1985) *Comprehensive Biotechnology Vol. III*, (Cooney, C.L. and Humphrey, A.E. eds). Pergamon Press, Oxford 271–294.
104. Covert, S.F. (1990) Ph.D. Thesis, University of Wisconsin, Madison (1990).
105. Aspinall, G.O. (1970) *Polysaccharides*, Pergamon Press, Oxford.
106. Timell, T.E. (1967) *Wood Sci. Technol.* **1**: 54–70.
107. Timell, T.E. (1982) *Wood Sci. Technol.* **16**: 83–122.
108. Wilkie, K.C.B. (1979) *Adv. Carbohydrate Chem. Biochem.* 215–264.
109. Sjostrom, E. (1981) *Wood Chemistry: Fundamentals and Applications*, Academic Press, New York.
110. Delmer, D.P. (1982) *CRC Handbook of Biosolar Resources*, (Zaborsky, O. ed.). 357–361.
111. Jeffries, T. (1990) *Biodegradation* **1**: 163–176.
112. Jiang, J., Cahng, H., Bhattahcharjee, S. and Kwoh, D. (1987) *J. Wood Chem. Technol.* **7**: 81–86.

113. Dekker, R.F.H. and Richards, G.N. (1976) *Adv. Carbohyd. Chem. Biochem.* **31**: 277–352.
114. Dekker, R.F.H. (1985) *Biosynthesis and Biodegradation of Wood Components*, (Higuchi, T. ed.). Academic Press, New York 505–533.
115. Takenishi, S. and Tsujisaka, Y. (1975) *Agric. Biol. Chem.* **39**: 2315–2323.
116. Dekker, R.F.H. and Richards, G.N. (1975) *Carbohyd. Res.* **39**: 97–114.
117. Dekker, R.F.H. and Richards, G.N. (1975) *Carbohyd. Res.* **42**: 107–123.
118. Hrmova, M., Biely, P. and Vrsanska, M. (1986) *Arch. Microbiol.* **144**: 307–311.
119. Royer, J.C. and Nakas, J.P. (1990) *Appl. Environ. Microbiol.* **56**: 2535–2539.
120. Leathers, T.D., Detroy, R.W. and Bothast, R.J (1986) *Biotechnol. Lett.* **8**: 867–872.
121. Wong, K.K.Y., Tan, L.U.L. and Saddler, J.N. (1988) *Microbiol. Rev.* **52**: 305–317.
122. Ujiie, M., Roy, C. and Yaguchi, M. (1991) *Appl. Environ. Microbiol.* **57**: 1860–1862.
123. Paice, M.G., Jurasek, L., Carpenter, M.R. and Smillie, L.B. (1978) *Appl. Environ. Microbiol.* **36**: 802–808.
124. Biely, P., Vrsanska, M. and Kratky, Z. (1980) *Eur. J. Biochem.* **112**: 313–321.
125. Morosoli, R., Roy, C. and Yaguchi, M. (1986) *Biochim Biophys Acta* **970**: 473–478.
126. Nakanishi, K., Arai, H. and Yasui, T. (1984) *J. Ferment. Technol.* **62**: 361–369.
127. Grepinet, O., Chebrou, M. and Beguin, P. (1988) *J. Bacteriol.* **170**: 4576–4581.
128. Grepinet, O., Chebrou, M. and Beguin, P. (1988) *J. Bacteriol.* **170**: 4582–4588.
129. Gilkes, N.R., Langsford, M.L., Kilburn, D.G., Miller, R.C. and Warren, R.A.J. (1984) *J. Biol. Chem.* **259**: 10455–10459.
130. Mondou, F., Shareck, F., Morosoli, R. and Kluepfel, D. (1986) *Gene* **49**: 323–329.
131. Yang, R.C., MacKenzie, C.R., Bilous, D., Seligy, V.L. and Narang, S.A. (1988) *Appl. Environ. Microbiol.* **54**: 1023–1029.
132. Yang, R.C., MacKenzie, C.R., Bilous, D. and Narang, S.A. (1989) *Appl. Environ. Microbiol.* **55**: 568–572.
133. Mannarelli, B.M., Evans, S. and Lee, D. (1990) *J. Bacteriol.* **172**: 4247–4254.
134. Hamamoto, T., Honda, H., Kudo, T. and Horikoshi, K. (1987) *Agric. Biol. Chem.* **51**: 953–955.
135. Boucher, F., Morosoli, R. and Durand, S. (1988) *Nuc. Acids Res.* **16**: 9874.
136. Van Doorslaer, V., Kersters-Hilderson, H. and DeBruyne, C.K. (1985) *Carbohyd. Res.* **140**: 342–346.
137. Matsuo, M. and Yasui, T. (1984) *Agric. Biol. Chem.* **48**: 1853–1860.
138. Rodionova, N.A., Tavobilov, I.M. and Bezborodov, A.M. (1983) *J. Appl. Biochem.* **5**: 300–312.
139. Despphande, V., Lachke, A., Mishra, C., Keskar, S. and Rao, M. (1986) *Biotechnol. Bioeng.* **28**: 1832–1837.
140. Matsuo, M. and Yasui, T. (1984) *Agric. Biol. Chem.* **48**: 1845–1852.
141. Deleyn, F., Claeyssens, M. and DeBruyne, C.K. (1982) *Meth. Enzymol.* **83**: 639–644.
142. Reese, E.T., Maguire, A. and Parrish, F.W. (1973) *Can. J. Microbiol.* **19**: 1063–1074.
143. Lee, S.F. and Forsberg, C.W. (1987) *Can. J. Microbiol.* **33**: 1011–1016.
144. Poutanen, K. (1988) *J. Biotechnol.* **7**: 271–282.
145. Kaji, A. (1984) *Adv. Carbohydr. Chem. Biochem.* **42**: 383–394.
146. Puls, J., Schmidt, O. and Granzow, C. (1987) *Enz. Microb. Technol.* **9**: 83–88.
147. Biely, P., Puls, J. and Schneider, H. (1985) *FEBS Lett.* **186**: 80–84.
148. Biely, P., MacKenzie, C.R., Puls, J. and Schneider, H. (1986) *Bio/Technology* **4**: 731–733.
149. Poutanen, K. and Sundberg, M. (1988) *Appl. Microb. Biotechnol.* **28**: 419–424.
150. Poutanen, K., Puls, J. and Linko, M. (1986) *Appl. Microb. Biotechnol.* **23**: 487–490.
151. Jeffries, T. (1985) *TIB* **3**: 208–212.
152. Jeffries, T. (1990) *Yeast Biotechnology and Biocatalysis* (Verachtert, H. and DeMot, R. eds). Marcel Dekker, New York, 349–394.
153. Kotter, P., Amore, R., Hollenberg, C.P. and Ciriacy, M. (1990) *Curr. Genet.* **18**: 493–500.
154. Grace, T.M., and Malcolm, E.W. (1989) *Pulp and Paper Manufacture, Vol. 5, Alkaline Pulping, Joint Textbook* Committee of the Paper Industry, TAPPI, Atlanta, Georgia.
155. Viikari, L., Kantelinen, A., Poutanen, K. and Ranua, M. (1990) *Biotechnology in Pulp and Paper Manufacture.* (Kirk, T.K. and Chang, H.M. eds), Butterworth-Heinemann Press, Boston 145–151.
156. Clark, T.A., McDonald, A.G., Senior, D.J. and Mayers, P.R. (1990) *Biotechnology in Pulp and Paper Manufacture*, (Kirk, T.K. and Chang, H.M. eds). Butterworth-Heinemann Press, Boston 153–167.

157. Skerker, P.S., Farrell, R.L. and Chang, H.M. (1991) Chlorine-Free Bleaching with Catazyme HS Treatment, *Proc. Int. Bleaching Conf., Swedish Assoc. Pulp and Paper Engineers*, Stockholm, June 11–14.
158. Pedersen, L.S., Nissen, A.M., Elm, D.D. and Choma, P.P. (1991) Bleach Boosting of Kraft Pulp Using Alkaline Hemicellulases, *Proc. Int. Bleaching Conf., Swedish Assoc. Pulp and Paper Engineers*, Stockholm, June 11–14.
159. duManoir, J.R., Hamilton, J., Senior, D.J., Bernier, R.L., Grant, J.E., Moser, L.E. and Dubelsten, P. (1991) Biobleaching of Kraft Pulps with Cellulase-Free Xyanases, *Proc. Int. Bleaching Conf., Swedish Assoc. Pulp and Paper Engineers*, Stockholm, June 11–14.
160. Tan, L.U.L., Yu, E.K.C.U., Loui-Seize, G.W. and Saddler, J.N. (1987) *Biotechnol. Bioeng.* **30**: 96–100.
161. Grant, R. (1991) *Pulp and Paper International* **33**: 61–63.
162. Paice, M.G., Bernier, R. and Jurasek, L. (1988) *Biotechnol. Bioeng.* **32**: 235–239.
163. Puls, J., Poutanen, K. and Lins, J.J. (1990) *Biotechnology in Pulp and Paper Manufacture.* (Kirk, T.K. and Chang, H.M. eds). Butterworth-Heinemann Press, Boston 183–190.
164. Senior, D.J., Mayers, P.R., Breuil, C. and Saddler, J.N. (1990) *Biotechnology in Pulp and Paper Manufacture* (Kirk, T.K. and Chang, H.M. eds). Butterworth-Heinemann Press, Boston 169–182.
165. Paice, M.G. and Lurasek, L. (1984) *J. Wood Chem. Technol.* **4**: 187–198.
166. Noe, P., Chevalier, J., Mora, F. and Comtat, J. (1986) *J. Wood Chem. Technol.* **6**: 167–184.
167. Olsen, W.L., Gallager, H.P., Burris, K.A., Bhattacharjee, S.S., Slocomb, J.P. and DeWitt, D. (1991) Enzymatic delignification of lignocellulosic materials, European Patent Application No. 90111620.2.
168. Adler, E. (1977) *Wood. Sci. Technol.* **11**: 169–218.
169. Harkin, J.M. (1967) Lignin–a natural polymeric product of phenol oxidation. In: *Oxidative Coupling of Phenols* (Taylor, W.I. and Battersby, A.R. eds). Marcel Dekker, New York, (1967) ch. 6.
170. Higuchi, T. (1990) *Wood Sci. Technol.* **24**: 23–63.
171. Sarkanen, K.V. and Ludwig, C.H. (eds). (1971) *Lignins. Occurrence, Formation, Structure and Reactions.* Wiley-Interscience, New York.
172. Burdsall, H.H. and Eslyn, W.E. (1974) *Mycotaxon* **1**: 123–133.
173. Raeder, U. and Broda, P. (1984) *Curr. Gen.* **8**: 499–506.
174. Jeffries, T.W., Choi, S. and Kirk, T.K. (1981) *Appl. Environ. Microbiol.* **42**: 290–296.
175. Keyser, P., Kirk, T.K. and Zeikus, J.G. (1978) *J. Bacteriol.* **135**: 790–296.
176. Kirk, T.K., Schultz, E., Conners, W.J., Lorenz, L.F. and Zeikus, J.G. (1978) *Arch. Microbiol.* **117**: 277–285.
177. Reid, I.D. (1979) *Can. J. Bot.* **57**: 2050–2058.
178. Bar-Lev, S.S. and Kirk, T.K. (1981) *Biochem. Biophys. Res. Comm.* **99**: 373–378.
179. Jäger, A., Croan, S. and Kirk, T.K. (1985) *Appl. Environ. Microbiol.* **50**: 1274–1278.
180. Lundquist, K. and Kirk, T.K. (1978) *Phytochemistry* **17**: 1676.
181. Shimada, M., Nakatsubo, F., Kirk, T.K. and Higuchi, T. (1981) *Arch. Microbiol.* **129**: 321–324.
182. Enoki, A. and Gold, M.H. (1982) *Arch. Microbiol.* **132**: 123–130.
183. Enoki, A., Goldsby, G.P. and Gold, M.H. (1980) *Arch. Microbiol.* **125**: 227–232.
184. Goldsby, G.P., Enoki, A. and Gold, M.H. (1980) *Arch. Microbiol.* **128**: 190–195.
185. Kamaya, Y. and Higuchi, T. (1984) *Wood Res.* **70**: 25–28.
186. Kirk, T.K. and Nakatsubo, F. (1983) *Biochim Biophys. Acta.* **756**: 376–384.
187. Nakatsubo, F., Reid, I.D. and Kirk, T.K. (1982) *Biochim Biophys. Acta.* **719**: 284–291.
188. Umezawa, T. and Higuchi, T. (1984) *Agric. Biol. Chem.* **48**: 1917–1921.
189. Umezawa, T. and Higuchi, T. (1985) *FEBS Lett.* **192**: 147–150.
190. Chen, C.-L., Chang, H.-m. and Kirk, T.K. (1982) *Holzforschung* **36**: 3–9.
191. Chen, C.-L., Chang, H.-m. and Kirk, T.K. (1983) *J. Wood Chem. Technol.* **3**: 35–57.
192. Chua, M.G.S., Chen, C.-L., Chang, H.-m. and Kirk, T.K. (1982) *Holzforschung* **36**: 165–172.
193. Kirk, T.K. and Adler, E. (1970) *Acta Chem. Scand.* **24**: 3379–3390.
194. Buswell, J.A. and Odier, E. (1987) *CRC Crit. Rev. Biotechnol.* **6**: 1–60.
195. Kirk, T.K. and Farrell, R.L. (1987) *Ann. Rev. Microbiol.* **41**: 465–505.
196. Kirk, T.K. and Shimada, M. (1985) *Biosynthesis and Biodegradation of Wood Components* (ed). Higuchi, T. Uni, Tokyo (1985) pp. 579–605.

197. Glenn, J.K., Morgan, M.A., Mayfield, M.B., Kuwahara, M. and Gold, M.H. (1983) *Biochem. Biophys. Res. Comm.* **114**: 1077–1083.
198. Gold, M.H., Kuwahara, M., Chiu, A.A. and Glenn, J.K. (1984) *Arch. Biochem. Biophys.* **234**: 353–362.
199. Tien, M. and Kirk, T.K. (1983) *Science (Washington DC)* **221**: 661–663.
200. Tien, M. and Kirk, T.K. (1984) *Proc. Natl Acad. Sci. USA* **81**: 2280–2284.
201. Kirk, T.K., Croan, S., Tien, M., Murtagh, K.E. and Farrell, R.L. (1986) *Enzyme Microb. Technol.* **8**: 27–32.
202. Leisola, M.S.A., Kozulic, B., Meusdoerffer, F. and Fiechter, A. (1987) *J. Biol. Chem.* **262**: 419–424.
203. Renganathan, V., Miki, K. and Gold, M.H. (1985) *Arch. Biochem. Biophys.* **241**: 304–314.
204. Farrell, R.L., Murtagh, K.E., Tien, M., Mozuch, M.D. and Kirk, T.K. (1989) *Enzyme Microb. Technol.* **11**: 322–328.
205. Glumoff, T., Harvey, P.J., Molinari, S., Goble, M. Frank, G., Palmer, J.M., Smit, J.D.G. and Leisola, M.S.A. (1990) *Eur. J. Biochem.* **187**: 515–520.
206. Troller, J., Smit, J.D.G., Leisola, M.S.A., Kallen, J., Winterhalter, K.H. and Fiechter, A. (1988) *Bio/Technology* **6**: 571–573.
207. Tien, M., Kirk, T.K., Bull, C. and Fee, J.A. (1986) *J. Biol. Chem.* **261**: 1687–1693.
208. Kuila, D., Tien, M., Fee, J.A. and Ondrias, M.R. (1985) *Biochemistry* **24**: 3394–3397.
209. Renganathan, V. and Gold, M.H. (1986) *Biochemistry* **25**: 1626–1631.
210. Andrawis, A., Johnson, K.A. and Tien, M. (1988) *J. Biol. Chem.* **263**: 1195–1198.
211. Marquez, L., Wariishi, H., Dunford, H.B. and Gold, M.H. (1988) *J. Biol. Chem.* **263**: 10549–10552.
212. Wariishi, H., Marquez, L., Dunford, H.B. and Gold, M.H. (1990) *J. Biol. Chem.* **265**: 11137–11142.
213. Leisola, M.S.A., Schmidt, B., Thanei-Wyss, U. and Fiechter, A. (1985) *FEBS Lett.* **189**: 267–270.
214. Umezawa, T., Shimada, M., Higuchi, T. and Kusai, K. (1986) *FEBS Lett.* **205**: 287–292.
215. Umezawa, T. and Higuchi, T. (1987) *FEBS Lett.* **218**: 255–260.
216. Kersten, P.J., Tien, M., Kalyanaraman, B. and Kirk, T.K. (1985) *J. Biol. Chem.* **260**: 2609–2612.
217. Hammel, K.E., Kalyanaraman, B. and Kirk, T.K. (1986) *Proc. Natl Acad. Sci. USA* **83**: 3708–3712.
218. Harvey, P.J., Schoemaker, H.E., Bowen, R.M. and Palmer, J.M. (1985) *FEBS Lett.* **183**: 13–16.
219. Schoemaker, H.E., Harvey, P.J., Bowen, R.M. and Palmer, J.M. (1985) *FEBS Lett.* **183**: 7–12.
220. Kirk, T.K., Tien, M., Kersten, P.J., Mozuch, M.D. and Kalyanaraman, B. (1986) *Biochem. J.* **236**: 279–287.
221. Snook, M.E. and Hamilton, G.A. (1974) *J. Am. Chem. Soc.* **96**: 860–869.
222. Hammel, K.E., Tien, M., Kalyanaraman, B. and Kirk, T.K. (1985) *J. Biol. Chem.* **260**: 8348–8353.
223. Renganathan, V., Miki, K. and Gold, M.H. (1986) *Arch. Biochem. Biophys.* **246**: 155–161.
224. Schoemaker, H.E. (1990) *Recl. Trav. Chim. Pays-Bas* **109**: 255–272.
225. Hammel, K.E., Kalyanaraman, B. and Kirk, T.K. (1986) *J. Biol. Chem.* **261**: 16948–16952.
226. Cavalieri, E. and Rogan, E. (1985) *Environ. Health Perspect.* **64**: 69–84.
227. Kersten, P.J., Kalyanaraman, B., Hammel, K.E., Reinhammar, B. and Kirk, T.K. (1990) *Biochem. J.* **268**: 475–480.
228. Millis, C.D., Cai, D., Stankovich, M.T. and Tien, M. (1989) *Biochemistry* **28**: 8484–8489.
229. Haemmerli, S.D., Leisola, M.S.A. and Fiechter, A. (1986) *FEMS Microbiol. Lett.* **35**: 33–36.
230. Hammel, K.E. and Moen, M.A. (1991) *Enzyme Microb. Technol.* **13**: 15–18.
231. Kondo, R., Limori, T., Imamura, H. and Nishida, T. (1990) *J. Biotechnol.* **13**: 181–188.
232. Leisola, M.S.A., Haemmerli, S.D., Waldner, R., Schoemaker, H.E., Schmidt, H.W.H. and Fiechter, A. (1988) *Cellulose Chem. Technol.* **22**: 267–277.
233. Kuwahara, M., Glenn, J.K., Morgan, M.A. and Gold, M.H. (1984) *FEBS Lett.* **169**: 247–250.
234. Paszczynski, P., Huynh, V.-B. and Crawford, R.L. (1985) *FEMS Microbiol. Lett.* **29**: 37–41.
235. Glenn, J.K. and Gold, M.H. (1985) *Arch. Biochem. Biophys.* **242**: 329–341.
236. Glenn, J.K., Akileswaran, L. and Gold M.H. (1986) *Arch. Biochem. Biophys.* **251**: 688–696.

237. Mino, Y., Wariishi, H., Blackburns, N.J., Loehr, T.M. and Gold, M.H. (1988) *J. Biol. Chem.* **263**: 7029–7036.
238. Paszczynski, A., Huynh, V.-B. and Crawford, R.L. (1986) *Arch. Biochem. Biophys.* **244**: 750–765.
239. Wariishi, H., Akileswaran, L. and Gold, M.H. (1988) *Biochemistry* **27**: 5365–5370.
240. Wariishi, H., Dunford, H.B., MacDonald, I.D. and Gold, M.H. (1989) *J. Biol. Chem.* **264**: 3335–3340.
241. Hammel, K.E., Tardone, P.J., Moen, M.A. and Price, L.A. (1989) *Arch. Biochem. Biophys.* **270**: 404–409.
242. Wariishi, H., Valli, K. and Gold, M.H. (1991) *Biochem. Biophys. Res. Comm.* **176**: 269–275.
243. Kersten, P.J. and Kirk, T.K. (1987) *J. Bacteriol.* **169**: 2195–2201.
244. Kersten, P.J. (1990) *Proc. Natl Acad. Sci. USA* **87**: 2936–2940.
245. Datta, A., Bettermann, A. and Kirk, T.K. (1991) *Appl. Environ. Microbiol.* **57**: 1453–1460.
246. Muheim, A., Leisola, M.S.A. and Schoemaker, H.E. (1990) *J. Biotechnol.* **13**: 159–167.
247. Kawai, S., Umezawa, T. and Higuchi, T. (1988) *Arch. Biochem. Biophys.* **262**: 99–110.
248. Kawai, S., Umezawa, T. and Higuchi, T. (1989) *Wood Res.* **76**: 10–16.
249. Morohoshi, N., Fujita, K., Wariishi, H. and Haraguchi, T. (1987) *Mokuzai Gakkaishi* **33**: 310–315.
250. Malmström, B.G., Andréasson, L.-E. and Reinhammar, B. (1975) Academic Press. New York.
251. Reinhammer, B. (1984) *Laccase*. CRC Press, Boca Raton, FL.
252. Andréasson, L.-E. and Reinhammar, B. (1976) *Biochim Biophys. Acta* **445**: 579–597.
253. Higuchi, T. (1989) In: *Plant Cell Wall Polymers Biogenesis and Biodegradation* (Lewis, N.G. and Paice, M.G. eds). ACS symp. series 399, American Chemical Society, Washington DC 482–502.
254. Galliano, H., Gas, G., Seris, J.L. and Boudet, A.M. (1991) *Enzyme Microb. Technol.* **13**: 478–482.
255. Gold, M.H., Cheng, T.M. and Mayfield, M.B. (1982) *Appl. Environ. Microbiol.* **44**: 996–1000.
256. Raeder, U. and Broda, P. (1985) *Lett. Appl. Microbiol.* **1**: 17–20.
257. Haylock, R., Liwicki, R. and Broda, P. (1985) *Appl. Environ. Microbiol.* **46**: 691–702.
258. Randall, T., Reddy, C.A. and Boominathan, K. (1991) *J. Bacteriol.* **173**: 776–782.
259. Alic, M., Mayfield, M.B., Akileswaran, L. and Gold, M. (1991) *Curr. Genet.* (in press).
260. Wang, J., Holden, D. and Leong, S. (1988) *Proc. Natl Acad. Sci. USA* **85**: 865–869.
261. Mellon, F., Litle, P. and Casselton, L. (1987) *Mol. Gen. Genet.* **210**: 352–357.
262. Tien, M. and Tu, C. (1987) *Nature* **326**: 520–523.
263. Andrawis, A., Pease, E., Kuan, I., Holzbaur, E. and Tien, M. (1989) *Biochem. Biophys. Res. Commun.* **162**: 673–680.
264. Smith, T., Schalch, H., Gaskell, J., Covert, S. and Cullen, D. (1988) *Nuc. Acids Res.* **3**: 1219.
265. Huoponen, K., Ollikka, P., Kalin, M., Walther, I., Mantsala, P. and Reiser, J. (1990) *Gene* **89**: 145–150.
266. deBoer, H.A., Zhang, Y., Collins, C. and Reddy, C.A. (1987) *Gene* **60**: 93–102.
267. Zhang, Y. (1987) Ph.D Thesis, Michigan State University.
268. Pribnow, D., Mayfield, M.B., Nipper, V.J., Brown, J.A. and Gold M.H. (1989) *J. Biol. Chem.* **264**: 5036–5040.
269. Godfrey, B.J., Mayfield, M.B., Brown, J.A. and Gold, M. (1990) *Gene* **93**: 119–124.
270. Pease, E.A., Andrawis, A. and Tien, M. (1989) *Biol. Chem.* **264**: 13531–13535.
271. Corcoran, L.M., Thompson, J.K., Walliker, D. and Kemp, D.J. (1988) *Cell.* **53**: 807–813.
272. Ono, B. and Ishino-Arao, Y. (1988) *Curr. Genet.* **14**: 413–418.
273. Rustehenko-Bulgac, E.P., Sherm, F. and Hicks, J.B. (1990) *J. Bacteriol.* **172**: 1276–1283.
274. Kinscherf, T. and Leong, S.A. (1988) *Chromosoma* **96**: 427–433.
275. Holzbaur, E. and Tien, M. (1988) *Biochem. Biophys. Res. Commun.* **155**: 626–633.
276. Brown, J., Alic, M. and Gold, M. (1991) *J. Bacteriol.* **173**: 4101–4106.
277. Walther, I., Kaelin, M., Reiser, J., Suter, F., Fritsche, B., Saloheimo, M., Leisola, M., Teeri, T., Knowles, J. and Fiechter, A. (1988) *Gene* **70**: 127–137.
278. Pease, E.A. and Tien, M. (1991) *Biocatalysts for Industry* (Dordick, J.S. ed.). Plenum Press, New York 115–135.
279. Saloheimo, M., Barajas, V., Niku-Paavola, M. and Knowles, J. (1989) *Gene* **85**: 343–351.

280. Johnson, T.M. and Li, J. (1991) Heterologous expression of an active lignin peroxidase from *Phanerochaete chrysosporium* using recombinant baculovirus. 75th Ann. Meeting FASEB, Atlanta, Georgia Abstract No. 6818.
281. Kojima, Y., Tsukuda, Y., Kawai, Y., Tsukamoto, A., Sugiura, J., Sakaino, M. and Kita, Y. (1990) *J. Biol. Chem.* **256**: 15224–15230.
282. Kirk, T.K. and Chang, H. (1981) *Enzyme Microb. Technol.* **3**: 189–196.
283. Eaton, D.C., Chang, H., Joyce, T.W., Jeffries, T.W. and Kirk, T.K. (1982) *TAPPI J.* **65**: 89–92.
284. Huynh, V.-B., Chang, H.-m. and Joyce, T.W. (1985) *TAPPI J.* **68**: 98–102.
285. Eaton, D.C. (1985) *Enzyme Microb. Technol.* 7: 194–196.
286. Bumpus, J.A., Tien, M., Wright, D. and Aust, S.D. (1985) *Science* **228**: 1434–1436.
287. Lamar, R.T. and Dietrich, D.M. (1990) *Appl. Environ. Microbiol.* **56**: 3093–3100.
288. Haemmerli, S.D., Leisola, M.S.A., Sangland, D. and Fiechter, A. (1986) *J. Biol. Chem.* **261**: 6900–6903.
289. Hammel, K.E. and Tardone, P.J. (1988) *Biochemistry* **27**: 6563–6568.
290. Sanglard, D., Leisola, M.S.A. and Fiechter, A. (1986) *Enzyme Microb. Technol.* **8**: 209–212.
291. Valli, K. and Gold, M.H. (1991) *J. Bacteriol* **173**: 345–352.

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