10 Application of PCR in Studying Lignocellulose Degradation by Basidiomycetes

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10.1 Introduction

Lignin, cellulose and hemicellulose are the major structural polymers of plant biomass comprising, respectively, 45%, 20–30% and 20–30% of the dry weight of woody plants (Kirk and Farrell, 1987; Boominathan and Reddy, 1992). Because of the importance of lignocellulose as a renewable resource for the production of paper products, feeds, chemicals and fuels, there has been a considerable amount of research in recent years on the fungal degradation of lignocellulose (Kirk and Farrell, 1987; Boominathan and Reddy, 1992). Among the fungi, wood-rot basidiomycetes are considered the most efficient degraders of lignocellulose. White-rot fungi completely mineralize the three major lignocellulose polymers to CO_2, whereas the brown-rot fungi efficiently decompose cellulose and hemicellulose components of wood but mineralize lignin only to a limited extent (Kirk and Farrell, 1987; Reddy, 1993; Reddy and D'Souza, 1994). Earlier research focused extensively on lignocellulose degradation by the white-rot basidiomycete *Phanerochaete chrysosporium* (Kirk and Farrell, 1987; Boominathan and Reddy, 1992; Cullen and Kersten, 1992; Gold and Alic, 1993). However, there has been a growing interest recently in studying lignocellulose degrading systems of other wood-rot basidiomycetes (Higuchi, 1993; de Jong *et al*., 1994; Hatakka, 1994; Tuor *et al.*, 1995).

An increasing number of researchers are currently utilizing molecular biological approaches to study fungal lignocellulose degradation (Cullen and Kersten, 1992; Reddy, 1993; Gold and Alic, 1993; Reddy and D'Souza, 1994). With the advent of the polymerase chain reaction (PCR) and its associated methodologies (Saiki *et al.*, 1985; Mullis and Faloona, 1987; Innis *et al.*, 1990;

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Steffan and Atlas, 1991; Bej and Mahbubani, 1992; Dieffenbach and Dveksler, 1995), many researchers are using PCR-based strategies to better understand the physiology and molecular biology of fungal lignocellulose degradation and this is the focus of this review. A broad overview of the PCR techniques is presented first and is followed by a review of the applications of PCR methodology. The latter includes the detection, isolation and characterization of genes involved in lignocellulose degradation as well as studies on the expression of these genes under varying environmental conditions.

10.2 Extraction of Nucleic Acids for PCR Amplification

10.2.1 From fungal mycelia

Most methods for DNA isolation from mycelia involve an initial lysis step using freeze–thaw cycles (Garber and Yoder, 1983), or by grinding the mycelia. Grinding is done with dry ice (Wahleithner *et al.*, 1996), liquid nitrogen (Raeder and Broda, 1988; Lee and Taylor, 1990; Graham *et al.*, 1994), liquid nitrogen and glass beads (Rao and Reddy, 1984), or glass beads only (van Vaerenbergh *et al.*, 1995). This is followed by the use of phenol/chloroform/isoamyl alcohol extraction of the DNA and precipitation at a high salt concentration with isopropanol or ethanol (Sambrook *et al.*, 1989). Gentle lysis of the mycelial cells by enzymatic hydrolysis (Black *et al.*, 1989; Li and Ljungdahl, 1994) results in more intact DNA, but the yield is lower (van Vaerenbergh *et al.*, 1995). Moreover, for use in PCR, mechanical shearing of the genomic DNA during isolation is not a major concern provided that the resulting DNA is ~10–20 kbp in size (Sogin, 1990; van Vaerenbergh *et al.*, 1995). However, in cases where the same DNA preparations are used for both PCR and library construction, the DNA needs to be relatively pure. This is accomplished by extraction with phenol (Ashktorab and Cohen, 1992), cetyltrimethylammonium bromide (Kim *et al.*, 1990; Graham *et al.*, 1994), or by using affinity chromatography (e.g. Qiagen tip-500 mini-columns from Qiagen Inc., Los Angeles) (Wahleithner *et al.*, 1996). In a recent study comparing various methods for extraction of DNA for PCR, it was reported that relatively pure DNA was needed even for PCR applications in order to remove inhibitors of *Taq* polymerase, especially from DNA samples of certain pigmented fungi (van Vaerenbergh *et al.*, 1995). These researchers also found that the use of glass beads for DNA isolation reduced the amount of contaminating polysaccharides and inhibitors, and suggested that the freeze–thaw-based method for DNA isolation was superior to the other methods tested.

Fungal RNA isolation for use in PCR is based on standard protocols using a guanidinium isothiocyanate step (Lucas *et al.*, 1977; Chirgwin *et al.*, 1979; Timberlake and Barnard, 1981; Sims *et al.*, 1988; Sambrook *et al.*, 1989), followed by ethanol precipitation in the presence of high concentration of

salt (Sambrook *et al.*, 1989; James *et al.*, 1992), or isolation using affinity-based systems (Li and Ljungdahl, 1994; Wahleithner *et al.*, 1996). Poly(A)-RNA is isolated from total RNA preparations using affinity column systems containing oligo-dT-cellulose (de Boer *et al.*, 1987; Sambrook *et al.*, 1989; Johnston and Aust, 1994a; Tempelaars *et al.*, 1994), or magnetic beads (Brooks *et al.*, 1993; Broda *et al.*, 1995; Lamar *et al.*, 1995).

10.2.2 From soil

Approaches used for the isolation of total DNA from soil, for PCR amplification of the fungal DNA component, are similar to those used for isolation and PCR amplification of soil bacterial DNA described previously (reviewed in Bej and Mahbubani, 1992; Picard *et al.*, 1992; Tebbe and Vahjen, 1993; Moré *et al.*, 1994; Selenska-Pobell, 1995; Van Elsas and Smalla, 1995). Johnston and Aust (1994b) used DNA extraction followed by PCR amplification for detecting fungal DNA in soils spiked with basidiomycetes. However, detection limits were low when compared to the use of template DNA isolated from pure cultures; this could be due to interference with the PCR amplification of the fungal DNA by the contaminating humic acids and DNA from other soil organisms (Tebbe and Vahjen, 1993; Tsai and Olson, 1993). In addition, clay in the soil can bind DNA and reduce the yield (Ogram *et al.*, 1987). Another approach used is first to extract fungal cells from the soil and then extract the DNA; however, this approach is found to be less efficient (Hilger and Myrold, 1991). Recently, Thorn *et al.* (1996) described a procedure in which they used a selective medium for isolating diverse groups of soil basidiomycetes. However, it is not known what proportion of the total basidiomycetes in soil were isolated by this method. DNA isolated from selected pure cultures of soil basidiomycetes, utilizing the procedure of Thorn *et al.* (1996), is currently being used in PCR for the isolation of laccase and *lip* gene sequences (C.A. Reddy and T.M. D'Souza, unpublished work).

10.3 PCR Amplification Methods used in Studies on Lignocellulose Degradation

A number of papers have been published recently on the use of PCR methodology for a variety of studies on lignocellulose degradation by basidiomycetes. These studies include the detection, isolation and characterization of partial or complete lignocellulose degradative genes, the analysis of allele segregation, and gene expression under varying environmental conditions. PCR procedures used in these studies include DNA-PCR, RT–PCR, competitive RT–PCR, and inverse PCR. In this section, we provide a brief description of these procedures.

10.3.1 DNA-PCR

Replication of both strands of a target DNA sequence using a reaction mixture consisting of a thermostable DNA polymerase, forward and reverse oligonucleotide primers, the four deoxynucleotide triphosphates (dNTPs), magnesium ions and an appropriate template DNA is the standard procedure used for DNA-PCR (Saiki *et al.*, 1985). The outcome is an exponential amplification of the specific target fragment, depending on the number of cycles of amplification performed (Saiki *et al.*, 1985; Mullis and Faloona, 1987; Saiki *et al.*, 1988; Vahey *et al.*, 1995). Recent technologies have refined this basic PCR procedure to make it simpler and more efficient, resulting in enhanced amplification fidelity, higher specificity and minimizing DNA contamination problems.

In studies on lignocellulose degradation by fungi, conventional DNA-PCR procedures have been used to isolate and characterize lignin peroxidase (Johnston and Aust, 1994b; Collins and Dobson, 1995; Rajakumar *et al.*, 1996; Schneider *et al.*, 1996), manganese peroxidase (Asada *et al.*, 1995), laccase (Giardina *et al.*, 1995; Wahleithner *et al.*, 1996; Yaver *et al.*, 1996; D'Souza *et al.*, 1996), cellobiohydrolase (Covert *et al.*, 1992; Tempelaars *et al.*, 1994), cellulase (Chow *et al.*, 1994) and xylanase (Li and Ljungdahl, 1994) genes, as well as to analyse the allelic segregation patterns of some of these genes (Gaskell *et al.*, 1992, 1994, 1995). The procedure has also been used to investigate the expression of lignocellulose degradative genes under varying environmental conditions (Covert *et al.*, 1992; Brooks *et al.*, 1993; Reiser *et al.*, 1993; Broda *et al.*, 1995; Gaskell *et al.*, 1995; Rajakumar *et al.*, 1996) as described in later sections in this chapter.

10.3.2 RT–PCR

RNA can also serve as a template for PCR amplification after conversion to cDNA (Todd *et al.*, 1987; Veres *et al.*, 1987). RNA-PCR, or RT–PCR (reverse transcriptase–PCR) as it is more popularly known, is a modified PCR procedure designed for analysing RNA transcripts. RT–PCR is more sensitive than other methods used for RNA analysis, such as Northern hybridization, S-1 nuclease analysis, RNase A protection assay and *in situ* hybridization (Kawasaki, 1990). The first step in this procedure is the synthesis of cDNA from RNA using reverse transcriptase. First strand cDNA is synthesized using either total RNA or mRNA as template. Random hexamers, the downstream primer, or oligo-dT can be used as primers, although the use of random hexamers consistently gives better amplification than the other two primers (Kawasaki, 1990). Since single RNA molecules can be amplified efficiently, even relatively crude RNA preparations can be used as the starting template for RT–PCR. The newly synthesized first strand cDNA is then used as a template for PCR, and the target fragment is amplified. In cases where the downstream primer is used for first-strand cDNA synthesis, it is necessary to add only the upstream

primer to the reaction mixture. However, both primers should be used for PCR in cases where random hexamers or oligo-dT primers were used in the first-strand cDNA synthesis (Ausubel *et al.*, 1995). Contaminating DNA serving as the template is a common problem encountered in RT–PCR. Solutions to this problem include the use of primers positioned in separate exons (Kawasaki, 1990), or RNase-free DNase I digestion to remove the contaminating DNA (Ausubel *et al.*, 1995).

In studies on lignocellulose degradation by basidiomycetes, the RT–PCR technique has been used in the isolation of lignin peroxidase (Rajakumar *et al.*, 1996), manganese peroxidase (Matsubara *et al.*, 1996), laccase (Giardina *et al.*, 1995) and cellobiohydrolase (Covert *et al.*, 1992) genes. The RT–PCR procedure has also been used in allele segregation studies (Gaskell *et al.*, 1995), and to investigate the expression of lignocellulose genes under different environmental conditions (Covert *et al.*, 1992; Brooks *et al.*, 1993; Johnston and Aust, 1994a; Broda *et al.*, 1995; Gaskell *et al.*, 1995; Lamar *et al.*, 1995; Bogan *et al.*, 1996b).

10.3.3 Competitive RT–PCR

Competitive RT–PCR is a technique that is commonly used for quantification of specific mRNA species (Gilliland *et al.*, 1990). This procedure involves co-amplification of a competing template that uses the same primers as those for the target cDNA, but the amplified products can be distinguished from each other as described below. Competitive templates can be either cDNA with a new restriction site, or genomic DNA containing an intron in the region to be amplified; the amplified product of the competitive template can then be differentiated from the amplified product of the target DNA based on size. PCR amplification with genomic DNA as the competitive template may not be as efficient as that using cDNA because of the size increase and changes in duplex melting temperature (Gilliland *et al.*, 1990). Target cDNA should be co-amplified with serial dilutions of competitor DNA of known concentration. Since a change in any of the variables (such as polymerase, dNTPs, Mg^{2+}, template DNA and primers) will affect the yield of target cDNA and the competitive template equally, relative ratios of the two should be preserved during amplification. The target cDNA concentration can then be estimated by direct scanning of ethidium bromide-stained gels or by measuring the radioactivity from incorporated radiolabelled dNTPs. Since the starting concentration of the competitive template is known, the initial concentration of the target cDNA can be determined fairly accurately. For example, it has been claimed that less than 1 pg of target cDNA from 1 ng of total mRNA can be accurately quantified using this procedure (Gilliland *et al.*, 1990).

The competitive RT–PCR procedure has been employed in studying the expression of lignin peroxidase (Stewart *et al.*, 1992; Lamar *et al.*, 1995), manganese peroxidase (Bogan *et al.*, 1996a) and cellobiohydrolase (Lamar *et al.*, 1995) genes.

10.3.4 Inverse PCR

DNA sequences that are outside of the target DNA sequence flanked by the PCR primers can also be amplified by a modification of the basic PCR procedure designated 'inverse PCR'. In this approach, the template is digested with a restriction enzyme that cuts outside the region to be amplified; the restricted template is then circularized by ligation, and the region outside the target sequence is amplified using primer sequences in the opposite orientation to those used for the initial PCR (Ochman *et al.*, 1988; Triglia *et al.*, 1988; Silver and Keerikatte, 1989). The inverse PCR method has been extremely useful in studying upstream and downstream regions of a target DNA segment, without the need to use conventional cloning techniques. It can also be used to prepare hybridization probes to identify and orient adjacent/overlapping clones isolated from a DNA library (Ochman *et al.*, 1990). The inverse PCR technique has been used in isolating the cellobiohydrolase gene (Tempelaars *et al.*, 1994).

10.4 Applications of PCR in Studies on Lignocellulose Degradation by Basidiomycetes

10.4.1 Detection, isolation, and characterization of genes involved in lignocellulose degradation

Wood-rot basidiomycetes are efficient degraders of the lignocellulose complex. The key enzymes involved are lignin peroxidases (LIP), manganese-dependent peroxidases (MNP), laccases, cellulases and xylanases. Several LIP (H1, H2, H6, H7, H8, and H10), and MNP (H3, H4, H5, and H9) isozymes produced by *Phanerochaete chrysosporium*, the prototype organism for studies on lignin degradation, have been described (Kirk and Farrell, 1987; Boominathan and Reddy, 1992; Cullen and Kersten, 1992; Gold and Alic, 1993). Recent studies have used PCR techniques to obtain a better understanding of the genes involved in lignocellulose degradation by *P. chrysosporium*, as well as a number of other wood-rot basidiomycetes.

Lignin peroxidase genes

Johnston and Aust (1994a) designed oligonucleotide primers H8I (forward) and H8II (reverse), corresponding to positions starting at 252 and 868 in the LIP H8 gene (Smith *et al.*, 1988) of *P. chrysosporium* BKM-F 1767, for the detection of LIP H8 mRNA by RT–PCR (Table 10.1). Sequencing of a 402 bp PCR product verified the presence of H8 mRNA under N-limited or N-sufficient culture conditions. In another study, Johnston and Aust (1994b) used the same primers to detect *P. chrysosporium* in soil by PCR amplification using total DNA isolated from soil as the template. This resulted in the amplification of a 616 bp PCR fragment. However, they report that the method for detecting *P. chrysosporium* BKM-F 1767 using the H8 gene

primers was not as sensitive as that using the internal transcribed spacer (ITS) region of fungal ribosomal DNA (see Table 10.6).

Collins and Dobson (1995) described a method for PCR amplification and cloning of *lip* gene sequences from four different white-rot fungi, including *P. chrysosporium* ATCC 32629, *Chrysosporium lignosum* PM1, *Coriolus versicolor* 290, and *Bjerkandera adusta* DSM 3375. Genomic DNA was used as the template, with oligonucleotide primers corresponding to the conserved amino acid motifs present in the distal and proximal histidine regions of several *P. chrysosporium* LIP isozymes (Table 10.1). PCR-amplified products, ranging between 600 and 700 bp, were sequenced and found to have a nucleotide similarity of 70–80%. One of the PCR products was also found to have a 90% similarity with *GLG5*, which encodes the LIP isozyme H10 (Gaskell *et al.*, 1991).

PCR-amplification procedures were also used to determine the possible presence of *lip* gene sequences in genomic DNA of two white-rot basidiomycetes *Ceriporiopsis subvermispora* and *Phanerochaete sordida*, which were not known to produce LIPs (Rajakumar *et al.*, 1996). Degenerate oligonucleotide primers were prepared corresponding to amino acid motifs surrounding an essential histidine residue highly conserved in LIPs but not in MNPs (Table 10.1). Three genomic clones, two from *C. subvermispora*, and one from *P. sordida*, were obtained after cloning of the PCR-amplified products. These clones showed 92.6–95.6% amino acid similarity to *lip A*, which encodes the LIP isozyme H8 in *P. chrysosporium* (Gaskell *et al.*, 1994).

Schneider *et al.* (1996) used oligonucleotide primers specific for the H8-encoding *LPOA* gene of *P. chrysosporium* BKM-F 1767 (Holzbaur and Tien, 1988) to PCR-amplify a 1590 bp fragment, designated *HG3*, from the genomic DNA of this organism. Cloning and sequencing of *HG3* showed that it is related to, but distinct from, *LPOA* (Holzbaur and Tien, 1988) and *GLG3* (Naidu and Reddy, 1990), both of which are known to encode LIP H8 in *P. chrysosporium*, and that *HG3* is possibly a non-allelic variant of the *P. chrysosporium* BKM-F 1767 LIP H8 gene.

Manganese-dependent peroxidase genes

Asada *et al.* (1995) amplified *mnp* gene-specific sequences from genomic DNA and cDNA of *Pleurotus ostreatus* using PCR. Synthetic 30- to 32-mer primers (sequences not shown) that match the exon sequences around the proximal and distal His residues of the MNP of *P. chrysosporium* were used to obtain PCR products pcr1 using genomic DNA as the template, and pcr2 using cDNA as the template. Both pcr1 and pcr2 were cloned and sequenced. A subsequent PCR, using cDNA as the template, and a 32-mer upstream primer (sequence not shown) corresponding to the N-terminal amino acid sequence of the purified MNP, and a 30-mer downstream primer (sequence not shown) corresponding to a region near the 3′-end of pcr2, yielded a third PCR product, designated pcr3. Comparison of the deduced amino acid

Table 10.1. Primers used for PCR amplification of lignin peroxidase-encoding genomic DNA and cDNA sequences.

Gene[a]	Organism	PCR[b] procedure	5′ Primer	3′ Primer	Amplified product (bp)[b]	Reference[b]
LPOA (LIP H8)	*Phanerochaete chrysosporium* BKM-F 1767	DNA-PCR[1] RT–PCR[2]	TGAGGCGCACGAGTCGATTCGTCT (H8I)	GACAAGCTCGAGCTCCTCGAACTC (H8II)	616[1], 402[2]	Johnston and Aust (1994a[1], b[2])
lip	*P. chrysosporium* ATCC 32699, *Chrysosporium lignorum* PM1, *Coriolus versicolor* 290, *Bjerkandera adusta* DSM 3375	DNA-PCR	T TTCCACGATGCCATCGC CT G T	T GCAGCAATAGAATGGGC C G CG G C	500–700	Collins and Dobson (1995)
lip	*P. sordida* HHB-8922, *Ceriporiopsis subvermispora* L14807	DNA-PCR	C A A GCCTCGTGCCCGAGCCCTTCC T T T G G	T AGCTGCGTCTCGATGAAGAACTG A A C	208–312	Rajakumar *et al.* (1996)
HG3 (LIP H8)	*P. chrysosporium* BKM-F 1767	DNA-PCR	ACTAAGCTTGAGCGGACATGGCCTT-CAAGCA	CGCTGGATCCTATGGCATCATTTAAGCACC	1590	Schneider *et al.* (1996)
lipA (LIP H8)	*P. chrysosporium* BKM-F 1767	RT–PCR	GCAGCTATCTCTCTCGCTCT	TCCCAGTTCTTCGTCGAG	683, 1012	Stewart *et al.* (1992); Lamar *et al.* (1995)
lipB (LIP H8)	*P. chrysosporium* BKM-F 1767	RT–PCR	GCAGCGATTTCCCTCGCACT	TCCCAGTTCTTCGTCGAG	683, 1006	Stewart *et al.* (1992)
GLG5, *lipC* (LIP H10)	*P. chrysosporium* BKM-F 1767	RT–PCR	GCTGTTCTTACCGCCGCTCT	TCCCAGTTCTTCGTCGAG	677, 999	Stewart *et al.* (1992); Lamar *et al.* (1995)
GLG4, *lipD* (LIP H2)	*P. chrysosporium* BKM-F 1767	RT–PCR	CAGCCCTCTCCGTCGCCCTG	TCCCAGTTCTTCGTCGAG	685, 1000	Stewart *et al.* (1992); Lamar *et al.* (1995)
lipE (LIP H8)	*P. chrysosporium* BKM-F 1767	RT–PCR	TCGCCGCGATCACCGTCGCC	TCCCAGTTCTTCGTCGAG	685, 1006	Lamar *et al.* (1995)
0282 (LIP H2)	*P. chrysosporium* BKM-F 1767	RT–PCR	GCGGCTATCTCTCTTGCGCT	TCCCAGTTCTTCGTCGAG	683, 1020	Stewart *et al.* (1992)

V4 (LIP H2)	*P. chrysosporium* BKM-F 1767	RT–PCR	GCCATCGCGATCTCTCCC	GACAAAGAATTGCGTATC	479, 694	Stewart *et al.* (1992)
LIG1 (LIP H8)	*P. chrysosporium* ME446 (ATCC 34541)	RT–PCR	GCCGCAATTTCTCTTGCTCTTTCCA [UMG#90019]	TACATCGAACCACGCGCACGATGATT [UMG#90020]	123[3], 126[4], 178[3], 179[4]	Brooks *et al.* (1993)[3]; Broda *et al.* (1995)[4]
LIG2 (LIP H8)	*P. chrysosporium* ME446 (ATCC 34541)	RT–PCR	CATCGCAATTTCGCCCGCCATG-GAGGCA	ACCTTCTGAACGAATGGCTTCTGGAGC	179, 222	Broda *et al.* (1992)
LIG3 (LIP H8)	*P. chrysosporium* ME446 (ATCC 34541)	RT–PCR	TATTGCCATCTCTCCTGCTATGGAGGCC	ATGTTAGGGTGGAAGTTGGGCTCGATG	126, 179	Broda *et al.* (1995)
LIG4 (LIP H8)	*P. chrysosporium* ME446 (ATCC 34541)	RT–PCR	GTGCGCCTGGTTCCCCATTCTGCAG	AATTGGTCTCGATAGTATCGAAGAC	222, 350	Broda *et al.* (1995)
LIG5[c] (LIP H2)	*P. chrysosporium* ME446 (ATCC 34541)	RT–PCR	TTGCCCCGACGGCGTGCACAC [UMG#90084]	GGTCTCGATCGAGGAGAAGGTAATGATC [UMG#90081]	235[3], 355[3], 222[4], 350[4]	Brooks *et al.* (1993)[3]; Broda *et al.* (1995)[4]
LIG6 (LIP ?)	*P. chrysosporium* ME446 (ATCC 34541)	RT–PCR	GACCTGCTCGAACGGCAAGGTCGTCC	CATGATAGAACCATCGGCGCCTCGC	222, 350	Broda *et al.* (1995)
lip	*P. chrysosporium* BKM-F 1767 (ATCC 24725)	DNA-PCR	GGAATTCCACGATGCNATCGC CT T [Oligo D]	T G CG G C AACTGCAGCAGCAATAGAATGTGC C G [Oligo P]	Not given	Reiser *et al.* (1995)
lipI (LIP H2)	*P. chrysosporium* ME446	RT–PCR	TATATGCCTCTGAGCTCCTG	ACGTCTGTCTAGAAGTATGC	683, 1020	Gaskell *et al.* (1995)
lip	*P. sordida* HHB-8922	RT–PCR	C A A GCCTCGTGCCCGAGCCCTTCC T T T G G	CGACTCAGAGCTACTTCTTGA	~200, ~310	Rajakumar *et al.* (1996)
lip	*C. subvermispora* L14807	RT–PCR	TTGTGCCGGAGCCGTTCC	GAAGAACTGGGAGTCGAA	No product	Rajakumar *et al.* (1996)
lipA (LIP H8)	*P. chrysosporium* BKM-F 1767	RT–PCR	TCCATCGCAATTTCGCCC	ACACGGTTGATGATTTGG	301, not given	Bogan *et al.* (1996b)
lipB (LIP H8)	*P. chrysosporium* BKM-F 1767	RT–PCR	GCTATTGCCATCTCTCCT	ACACGAGCGATGATCTGG	301, not given	Bogan *et al.* (1996b)
lipC (LIP H10)	*P. chrysosporium* BKM-F 1767	RT–PCR	GCCATCGCTATCTCTCCC	ACACGGTCGATGATTTGG	298, not given	Bogan *et al.* (1996b)

continued overleaf

Table 10.1 continued.

Gene[a]	Organism	PCR[b] procedure	5′ Primer	3′ Primer	Amplified product (bp)[b]	Reference[b]
lipD (LIP H2)	*P. chrysosporium* BKM-F 1767	RT–PCR	TCCATCGCTATCTCGCCC	ATGCGAGCGAGAACCTGA	301, not given	Bogan *et al.* (1996b)
lipE (LIP H8)	*P. chrysosporium* BKM-F 1767	RT–PCR	TCCATCGCCATCTCGCCC	ACGCGGGCGATGATCTGG	301, not given	Bogan *et al.* (1996b)
lipF (LIP ?)	*P. chrysosporium* BKM-F 1767	RT–PCR	TGCCCTTGAGTCTCAAGG	ACGCGAGAGATGATGTGG	301, not given	Bogan *et al.* (1996b)
lipG (LIP H8)	*P. chrysosporium* BKM-F 1767	RT–PCR	TCGATCGCCATCTCGCCC	ACACGCTCGATGAGCTGG	301, not given	Bogan *et al.* (1996b)
lipH (LIP H8)	*P. chrysosporium* BKM-F 1767	RT–PCR	GCAATTGCCATCTCGCCC	ACACGGTTAATGAGCTGG	301, not given	Bogan *et al.* (1996b)
lipI (LIP H2)	*P. chrysosporium* BKM-F 1767	RT–PCR	TCTATCGCTATCTCTCCC	ACACGGCTGATGATTTGA	301, not given	Bogan *et al.* (1996b)
lipJ (LIP H2)	*P. chrysosporium* BKM-F 1767	RT–PCR	GCCATCGCGATCTCTCCC	ACCCGAGCCAGGATTTGA	298, not given	Bogan *et al.* (1996b)

[a]Lignin peroxidase isozymes encoded by the given *lip* genes (Kirk and Farrell, 1987; Boominathan and Reddy, 1992; Cullen and Kersten, 1992; Gold and Alic, 1993) are given in parentheses.

[b]Superscripts in the 'PCR procedure' and 'Amplified product' columns refer to the corresponding superscripts in the 'Reference' column.

[c]The upstream and downstream sequences of the primers for *LIG5* were erroneously switched in Table 1 of Broda *et al* (1995). These sequences are shown correctly in this Table and in Brooks *et al* (1993).

sequence of pcr3 to the N-terminal amino acid sequences of MNPs revealed that it was part of an MNP gene. Using the cassette–primer PCR technique and a high fidelity Takara EX *Taq* polymerase, full-length sequences of genomic DNA and cDNA of the *mnp* gene were then obtained, cloned, sequenced and analysed. Their results showed that the *P. ostreatus mnp* gene is more closely related to the *P. chrysosporium lip* genes, with respect to sequence similarity and intron/exon organization, than to the *mnp* genes of this organism.

Full-length *mnp* genes were also obtained using PCR with primers (Table 10.2) corresponding to the region of the translational start and stop codons of *mnp* genes of *P. chrysosporium* BKM-F 1767 (Bogan *et al.*, 1996a). These PCR-amplified MNP gene products served as templates in competitive RT–PCR experiments to analyse *mnp* transcripts of *P. chrysosporium* BKM-F 1767 during bioremediation of soils contaminated with polyaromatic hydrocarbons (PAH) (described in section 10.4.3).

Matsubara *et al.* (1996) performed RT–PCR of poly(A)-RNA from the lignin-degrading deuteromycete IZU-154. DNA primers were synthesized corresponding to amino acid sequences around distal and proximal histidine residues in *mnp* genes of *P. chrysosporium* (Table 10.2). The resulting PCR fragments, approximately 400 bp in size, were isolated and labelled with digoxigenin using the DIG DNA labelling kit (Boehringer Mannheim), and were used to screen the cDNA library of IZU-154. Eight positive clones were obtained and separated into two groups based on restriction enzyme mapping and partial nucleotide sequences. Two cDNAs (*IZ-MnP1* and *IZ-MnP2*), representative of the two groups, were further analysed. The coding sequences of *IZ-MnP1* and *IZ-MnP2* were 1152 bp (384 amino acids) and 1155 bp (385 amino acids), respectively, and showed 96.2% nucleotide and 95.1% amino acid similarities to each other. Also, *IZ-MnP1* showed 72.5% nucleotide and 79% amino acid similarity to *P. chrysosporium mnp1* cDNA (Pribnow *et al.*, 1989).

Laccase genes

Laccase, in addition to its role in lignin biodegradation (Bourbonnais and Paice, 1990; Hatakka, 1994), also has important applications in the bleaching of wood pulp (Bourbonnais and Paice, 1992) and the degradation of phenolic compounds (Bollag *et al.*, 1988; Roy-Arcand and Archibald, 1991). The structure and function of fungal laccases were reviewed by Thurston (1994). A number of researchers have used PCR as a tool in their studies on the laccase genes of wood-rotting basidiomycetes (Giardina *et al.*, 1995, 1996; Yaver *et al.*, 1996; Wahleithner *et al.*, 1996; D'Souza *et al.*, 1996) and these studies are reviewed in this section.

Giardina *et al.* (1995) used RT–PCR and the rapid amplification of cDNA ends (RACE) technique (Frohman *et al.*, 1988) to amplify partial cDNA fragments encoding the laccase *pox1* gene from the white-rot basidiomycete *Pleurotus ostreatus*. After first-strand cDNA synthesis using

Table 10.2. Primers used for PCR amplification of manganese peroxidase-encoding genomic DNA and cDNA sequences.

Gene[a]	Organism	PCR procedure	5′ Primer	3′ Primer	Amplified product (bp)	Reference
mnp-1 (MNP H4)	*Pleurotus ostreatus* IFO 30160	DNA-PCR	Not given	Not given	Not given	Asada *et al.* (1995)
mnp-1 (MNP H4)	*P. chrysosporium* BKM-F 1767 (ATCC 24725)	DNA-PCR	GCAATGGCCTTCGGTTCTCT	TTAGGCAGGGCCATCGAACT	Not given	Bogan *et al.* (1996a)
mnp-2 (MNP ?)	*P. chrysosporium* BKM-F 1767 (ATCC 24725)	DNA-PCR	CAGATGGCCTTCAAGTCCCT	TTATGCGGGACCGTTGAACT	Not given	Bogan *et al.* (1996a)
mnp-3 (MNP H3)	*P. chrysosporium* BKM-F 1767 (ATCC 24725)	DNA-PCR	GCACTCAAGCCAGCGCAATG	TGTCCGGCGCGTCAGACTTA	Not given	Bogan *et al.* (1996a)
MNP	Fungus IZU-154, *P. chrysosporium* ME446	RT–PCR	ATCCGCCTCACCTTCCACGA	GCGACGGAGTGGGAGGCGAG	~400	Matsubara *et al.* (1996)
mnp1 (=*mnp-2*) (MNP ?)	*P. chrysosporium* ME446 (ATCC 34541)	RT–PCR	TCCGGTCAACGGCTTGGTATTCCAG	GCGATCGTCTTGTTCGGGCGGCCAG	517, 676	Broda *et al.* (1996)
mnp-1 (MNP H4)	*P. chrysosporium* BKM-F 1767 (ATCC 24725)	RT–PCR	CAGACGGTACCCGCGTCACC	AGTGGGAGCGGCGACATCAC	Not given	Bogan *et al.* (1996a)
mnp-2 (MNP ?)	*P. chrysosporium* BKM-F 1767 (ATCC 24725)	RT–PCR	CCGACGGCACCCGCGTCAGC	CGAGCGGGAGCGGCGACGCC	Not given	Bogan *et al.* (1996a)
mnp-3 (MNP H3)	*P. chrysosporium* BKM-F 1767 (ATCC 24725)	RT–PCR	CCGACGGTACCAAGGTCAAC	AGCGGCAGCGGCGACGCGAC	Not given	Bogan *et al.* (1996a)

[a]Manganese peroxidase isozymes encoded by the given *mnp* genes (Kirk and Farrell, 1987; Boominathan and Reddy, 1992; Cullen and Kersten, 1992; Gold and Alic, 1993) are shown in parentheses.

reverse transcriptase (RT) and the RT primer, another primer was used to amplify a 750 bp cDNA fragment corresponding to the 3′-end of the *pox1* gene (Table 10.3). A second PCR was carried out using two new primers and the first-strand cDNA as template to amplify a 900 bp cDNA fragment corresponding to the 5′-end of the *pox1* gene. Except the RT primer, the primers were derived from the genomic DNA sequence determined earlier for the *pox1* gene (Table 10.3). Together the two PCR fragments obtained accounted for the entire *pox1* cDNA. The deduced amino acid sequence of *pox1* showed 45–63% similarity with the laccase genes of *Coriolus hirsutus*, basidiomycete PM1 (CECT 2971), *Phlebia radiata* and *Agaricus bisporus*, but only 20–27% similarity with the laccase genes of *Neurospora crassa*, *Aspergillus nidulans* and *Cryphonectria parasitica* (Giardina *et al.*, 1995). In order to determine whether *pox1* and *pox2* (another *P. ostreatus* laccase gene) were allelic or not, two separate PCR amplification experiments were performed using the genomic DNA from six monokaryotic isolates as the templates. The two primer sets were located at identical positions on both *pox1* and *pox2* sequences, thus resulting in the amplification of 570 bp fragments (Table 10.3). However, the two products were distinguishable because of the presence of a unique *Hin*dIII site in *pox1*, and a unique *Bam*HI in *pox2*. The results showed that the two genes segregated together into monokaryons and hence were non-alleles (Giardina *et al.*, 1995).

In a subsequent study, Giardina *et al.* (1996) used RT–PCR to synthesize the entire laccase *pox2* cDNA sequence, as well as the genomic equivalent of the *pox2* gene. RT–PCR was done using mRNA and the oligonucleotide primer dT-*Not*I (Table 10.3). This was followed by PCR with the high fidelity *Pfu* polymerase, using the primer pairs O1/O2, LAC1/LAC2, and LAC3/dT*Not*I (Table 10.3), at annealing temperatures of 50°C, 60°C and 53°C, respectively. To amplify the 3′-end of the *pox2* gene, primer pairs LAC3/LAC4 (Table 10.3) were used with *Pfu* polymerase, genomic DNA as template, and an annealing temperature of 60°C. The *pox2* cDNA had 84% nucleotide and 90% amino acid similarity with the *pox1* cDNA (Giardina *et al.*, 1996).

Yaver *et al.* (1996) used RT–PCR and two degenerate oligonucleotide primers (Table 10.3), one based on the N-terminal sequence of laccase and the other based on the C-terminal sequence of the *C. hirsutus* gene, to amplify a laccase gene-specific PCR product from *Trametes villosa* using first-strand cDNA synthesized from poly(A)-RNA of xylidine-induced cells. The first-strand cDNA was synthesized using reverse transcriptase and a commercial cDNA synthesis kit (Gibco BRL). The PCR product was then used as a probe to screen a cDNA library of *T. villosa* and obtain more than 100 positive clones. Out of these, the longest clone (LCC cDNA) was sequenced and found to have an identity of 90% with the sequence of the *C. hirsutus* laccase.

A 220 bp PCR-amplified fragment, isolated from genomic DNA of the phytopathogenic fungus *Rhizoctonia solani* with oligonucleotide degenerate primers, was used to isolate laccase gene sequences from a *R. solani* genomic

Table 10.3. Primers used for PCR amplification of laccase-encoding genomic DNA and cDNA sequences.

Gene[a]	Organism	PCR procedure	5′ Primer	3′ Primer	Amplified product (bp)	Reference[a]
Laccase (*3′-pox1*)	*Pleurotus ostreatus* (str. *Florida*)	RT–PCR	CGCAGATCCCAACTTGGGATCGACTGGCTT [03]	AATTCGCGGCCGCTTTTTTTTTTTTTTT [04]	750	Giardina *et al.* (1995)
Laccase (*5′-pox1*[1], *5′-pox2*[2])	*Pleurotus ostreatus* (str. *Florida*)	RT–PCR	ATGTTTCCAGGCGCACGG [01]	CCAGTCGATCCCAAGTTGGG [02]	900	Giardina *et al.* (1995[1], 1996[2])
Laccase (*pox1*)	*Pleurotus ostreatus* (str. *Florida*)	DNA-PCR	GCTGGCACGTTCTGTAAG [05]	TCTGCCTCGATGACCTGC [06]	570	Giardina *et al.* (1995)
Laccase (*pox2*)	*Pleurotus ostreatus* (str. *Florida*)	DNA-PCR	GCTGGAACGTTTTGTAAG [07]	TCTGCTTCAATGACGAGC [09]	570	Giardina *et al.* (1995)
Laccase (*3′-pox2*)	*Pleurotus ostreatus* (str. *Florida*)	RT–PCR	CGCTCTAGACTCGTCATTGAAGCAGATG [LAC1]	GCGCTGCAGCTAAGCTATGCCACCTTTGTC [LAC2]	842	Giardina *et al.* (1996)
Laccase (*3′-pox2*)	*Pleurotus ostreatus* (str. *Florida*)	RT–PCR	CATCCATTCCATCTTCACGGC [LAC3]	AATTCGCGGCCGCTTTTTTTTTTTTTTT [dt-*Not*I] [=04, see above]	434	Giardina *et al.* (1996)
Laccase (*3′-pox2*)	*Pleurotus ostreatus* (str. *Florida*)	DNA-PCR	CATCCATTCCATCTTCACGGC [LAC3]	CGTCGGAGATGAGGTTC [LAC4]	Not given	Giardina *et al.* (1996)

Laccase	*Trametes villosa*	RT–PCR	ACCAGNCTAGACACGGGNTGAGATACT-GACGNGAAGCGGACTTGCTGGTCACT-ATCTTCGAAGATCTCG	CGCGGCCGCTAGGATCCTCACAATGGCCAA-GTCTCTGCCTCGACCTTC	Not given	Yaver *et al.* (1996)
Laccase	*Rhizoctonia solani*	DNA-PCR	T ATCCATTGGCATGGNTTTTTTCAA A C C C C G	GTGNCAATGATACCAGAANGT A GTG G A	220	Wahleithner *et al.* (1996)
Laccase	*Fomes fomentarius* TVJ-93-7-T, *Ganoderma lucidum* (strains 58537 and 103561), *Gloeophyllum trabeum* Madison-617-R, *Grifola frondosa*, *Lentinula edodes* RA-3-2-E, *Lentinus tigrinus*, *Phlebia brevispora* HHB-7099-Sp, *Phlebia tremellosa* FP-101416, *Pleurotus ostreatus* NRRL 2366, *Trametes* (*Coriolus*) *versicolor* ATCC 12679	DNA-PCR	CATTGGCATGGNTTTTTTCA C C C C	GTGGCTATGATACCAGAANGT A A G G A	144–217	D'Souza *et al.* (1996)

[a]Superscripts in the 'Gene' column refer to the corresponding superscripts in the 'Reference' column.

DNA library (Wahleithner *et al.*, 1996). The primers corresponded to two amino acid sequences conserved among laccases (IHWHGFFQ and TFWYHSH; Table 10.3). The sequence of the cloned PCR fragment had 60% similarity to the corresponding region of a *C. hirsutus* laccase gene (Kojima *et al.*, 1990). Sequencing of 15 other PCR clones established the presence of three distinct laccase genes, designated *lcc1*, *lcc2*, and *lcc3*. Using the cloned PCR fragment as the probe, nine genomic clones were isolated from the *R. solani* genomic library.

D'Souza *et al.* (1996) used primers (Table 10.3), based on the conserved sequences around the two pairs of histidines in the N-terminal copper-binding regions of known basidiomycete laccases, to rapidly isolate laccase gene-specific sequences from different wood-rot basidiomycetes. Genomic DNA was isolated from each of the wood-rot fungi and used as the template in PCR. Of the 11 white-rot and brown-rot fungi (belonging to nine genera) used for the amplification of the laccase gene-specific PCR products, eight gave amplification products of approximately 200 bp. One strain, *Gloeophyllum trabeum* Mad-617-R, a brown-rot fungus, gave a 144 bp PCR product. No PCR products were obtained for *Fomes fomentarius* and *Pleurotus ostreatus*, two white-rot fungi which are known to produce laccases (Hatakka, 1994). However, Southern hybridization of restriction enzyme-digested genomic DNA of *F. fomentarius* and *P. ostreatus*, using the *G. trabeum* 144 bp PCR product as the probe, showed hybridization bands indicating that laccase genes are present in these two strains. Several of the white-rot fungi tested gave two PCR products, one of 144 bp, and the other of the expected 200 bp. Sequencing of the larger PCR product revealed a size range of 197–217 bp; the smaller PCR products all were 144 bp in size (D'Souza *et al.*, 1996). All the PCR products sequenced had a high degree of similarity to corresponding regions of previously published laccase gene sequences. Alignment of the predicted amino acid sequences of the PCR-amplified products showed high similarities between the laccase gene segments of the wood-rotting fungi and lower similarities to those of *Aspergillus nidulans* and *Neurospora crassa*, two laccase producing non-wood rotters (Fig. 10.1). Following up on the demonstration of a laccase gene-specific PCR product from the genomic DNA of the brown-rot fungus *G. trabeum*, D'Souza *et al.* (1996) demonstrated for the first time the presence of laccase activity in cultures of this and other brown-rot fungi. Using the approach described above, a laccase gene-specific 199 bp PCR product was recently isolated and sequenced from a soil basidiomycete, AX-1 (Srinivasan *et al*., 1996). The laccase gene fragment of AX-1 showed a high degree of nucleotide similarity (62–94%) to the corresponding portions of laccase genes from wood-rotting basidiomycetes.

Cellulase genes

A large number of microorganisms degrade cellulose in nature; of these, fungi are perhaps the most studied. Cellulolytic enzymes include the

Fig. 10.1. Alignment of the predicted amino acid sequences of the PCR-amplified laccase gene products (D'Souza *et al.,* 1996). The abbreviations for the fungal strains are: *Tv, Trametes versicolor*; *Pb, Phlebia brevispora*; Gl_{103561} and Gl_{58537}, *Ganoderma lucidum* strains 103561 and 58537; *Le1* and *Le2, Lentinula edodes* PCR products 1 and 2; *Lt, Lentinus tigrinus*; *Gt, Gloeophyllum trabeum*; *Tvi, Trametes villosa*; *Po, Pleurotus ostreatus*; PM1, unidentified basidiomycete; *Ch, Coriolus hirsutus*; *Cv, Coriolus versicolor*; *Pr, Phlebia radiata*; *Ab, Agaricus bisporus*; *An, Aspergillus nidulans*; *Nc, Neurospora crassa*. Sequences from the latter nine organisms were taken from published literature (see D'Souza *et al.,* 1996). Alignment was done using the GENEPRO program (Riverside Scientific Enterprises). The program introduces gaps where necessary to optimize the alignments. Amino acid positions with a ≥ 50% match are highlighted. Invariant amino acid positions are shown in bold.

hydrolytic cellulases and oxidative cellulases. Hydrolytic cellulases consist of three classes: endoglucanases (EG), cellobiohydrolases (CBH) and β-glucosidases. Oxidative cellulases consist of two classes: cellobiose quinone oxidoreductase (cellobiose dehydrogenase) and cellobiose oxidase (Eriksson *et al.*, 1990; Béguin and Aubert, 1995). Fungal and bacterial cellulases share similar functional characteristics, including a catalytic core, a conserved cellulose-binding terminal region, and an intervening, highly glycosylated hinge region (Knowles *et al.*, 1987; Gilkes *et al.*, 1991; Bayer *et al.*, 1996). A brief review of the use of PCR methodology for studying the cellulases of ligninolytic fungi is presented here.

Covert *et al.* (1992) characterized three structurally related CBH genes (*cbh1-1*, *cbh1-2* and *cbh1-3*) from *P. chrysosporium* which were closely related to the *Trichoderma reesei cbh-1* gene (Shoemaker *et al.*, 1983). Full-length cDNA species encoding *P. chrysosporium cbh1-1*, *cbh1-2* and *cbh1-3* were amplified by PCR, cloned, and sequenced. RT–PCR was carried out using total RNA isolated from *P. chrysosporium* BKM-F 1767 cellulolytic

cultures using downstream primers (Table 10.4) to synthesize first-strand cDNA of *cbh1-1* and *cbh1-3*. Then, upstream primers were used to amplify the cDNA for these two genes. The cDNA *cbh1-2* was amplified by DNA-PCR employing double-stranded cDNA (synthesized using an oligo-dT primer) as the template and two specific primers (Table 10.4) (Covert *et al.*, 1992). A highly conserved region, assumed to be the catalytic site, within the *P. chrysosporium* cDNAs *cbh1-1*, *cbh1-2* and *cbh1-3* was 80%, 69% and 80% similar, respectively, to the corresponding region within the *T. reesei cbh-1* gene. However, analysis of the *P. chrysosporium cbh1-1* gene revealed that it was different from other fungal cellulase genes because it did not contain the hinge or tail regions. Transcription studies on these three genes were also carried out using competitive RT–PCR (described in Section 10.4.3).

Two allelic variants of the *P. chrysosporium* ME-446 *cbhII* gene were isolated from a cDNA library after screening with a *T. reesei cbhII* gene probe (Tempelaars *et al.*, 1994). Characterization of these cDNAs by restriction enzyme analysis, Southern hybridization analysis, and partial sequencing using the dideoxy chain-termination method, revealed that they were very similar, and in the cellulose-binding domain had a 65% amino acid similarity to the corresponding region of the *T. reesei cbhII* gene. The results also revealed that the entire *cbhII* gene is present on a *Pst*I genomic fragment. Therefore, inverse PCR (Ochman *et al.*, 1990) was used to amplify a 1.1 kbp fragment from *Pst*I-digested and religated genomic DNA, using two primers (Table 10.4) deduced from the *cbhII* cDNA sequence, and the high fidelity *Pfu* polymerase. The inverse PCR was followed by conventional DNA-PCR using the 1.1 kbp PCR-amplified DNA fragment as the template and two primers, based on the DNA sequence adjacent to the *Pst*I restriction site. The resulting 2.3 kbp PCR-amplified genomic *cbhII* fragment was cloned, sequenced, and analysed. Sequence comparison showed that both the genomic and cDNA clones represent two distinct classes (type 1 and type 2), which were later shown to be allelic variants (Tempelaars *et al.*, 1994).

Chow *et al.* (1994) isolated two allelic forms of a cellulase (endoglucanase) CEL3-encoding cDNA from a cDNA library of *A. bisporus*, an important commercial mushroom. The cDNA library was constructed in lambda ZAP-PII (Raguz *et al.*, 1992) from cDNA synthesized from a cellulose-grown mycelial culture. A sample of this cDNA library was excised *in vivo* as pBluescript plasmids with R408 helper phage (Short *et al.*, 1988), and total plasmid DNA was isolated from the bacterial culture harbouring these plasmids. PCR amplification was performed on the total plasmid DNA using a 20-mer degenerate oligonucleotide (OL60B1, Table 10.4), which is complementary to a region from a 19 kDa CNBr CEL3-cleaved peptide, as the reverse primer, and the T7 primer (Table 10.4) which corresponds to a region 5′ of the insertion site in the pBluescript plasmid, as the forward primer. Three PCR amplified products, 400 bp, 600 bp and 1.1 kbp in size, were obtained. Northern hybridization studies showed that the 400 bp PCR

Table 10.4. Primers used for PCR amplification of cellulase-encoding genomic DNA and cDNA sequences.

Gene	Organism	PCR procedure	5′ Primer	3′ Primer	Amplified product (bp)[a]	Reference[a]
cbh1-1	*P. chrysosporium* BKM-F 1767 (ATCC 24725)	RT–PCR	GAGAATTCTGAAACCGCTACACATT	TGAATTCCACCAATATTCACGCAGG	Not given	Covert *et al.* (1992)
cbh1-2	*P. chrysosporium* BKM-F 1767 (ATCC 24725)	DNA-PCR	GAGAATTCGAGGTTGAGACGCGATA	TTGTGACCCCAGCATTAGATACA	Not given	Covert *et al.* (1992)
cbh1-3	*P. chrysosporium* BKM-F 1767 (ATCC 24725)	RT–PCR	GAGAATTCTACCGTCTGCTCACACT	GAGAATTCCTTAGTAGCACTGGGA	Not given	Covert *et al.* (1992)
cbh1-1	*P. chrysosporium* BKM-F 1767 (ATCC 24725)	Competitive RT–PCR	CACAGTGTGTCCAAGGGATGTC [pr6]	CAAAAGCCCGTGTTGGAGGT [pr7]	221, 274	Covert *et al.* (1992)
cbh1-2	*P. chrysosporium* BKM-F 1767 (ATCC 24725)	Competitive RT–PCR	GCCGATGGTGGACTTGC [Ps1]	CGTGGTACCAAGCCAGCCTT [pr5]	132, 197	Covert *et al.* (1992)
cbh1-3	*P. chrysosporium* BKM-F 1767 (ATCC 24725)	Competitive RT–PCR	GCTAAGTACGGTACCGGCTA [pr3]	GCAGTCGCTTCCGGAGCAGC [pr2]	226, 286	Covert *et al.* (1992)
cbhII	*P. chrysosporium* ME446 (ATCC 34541)	Inverse PCR	GACGTCTAGAGGCTGGTGATAACGG	ATCAAGCTTGCCGGCTTCGGTACCC	1100	Tempelaars *et al.* (1994)
cbhII	*P. chrysosporium* ME446 (ATCC 34541)	DNA-PCR	GCGAATTCCACATTCCAGCATTTCAT-CCGTTG	CGCTGCAGAGATGCTAACCGAATTT-CAACCGG	2300	Tempelaars *et al.* (1994)
cbh1.1	*P. chrysosporium* ME446 (ATCC 34541)	DNA-PCR	ACAATGTTCCGCACTGCTACTT [G36]	AGGGTGCCCGCGGAGGTGCC [G32]	Not given[1], 840[2], 950[2]	Tempelaars *et al.* (1994)[1], Broda *et al.* (1995)[2]

Table 10.4 continued

Gene	Organism	PCR procedure	5′ Primer	3′ Primer	Amplified product (bp)[a]	Reference[a]
cbh1.2	*P. chrysosporium* ME446 (ATCC 34541)	DNA-PCR	CACTCCTCGCATTCACTTGTCT [G34]	CTGCCGGTCTCGGTCCAGTTGC [G35]	Not given[1], 560[2], 840[2]	Tempelaars *et al.* (1994)[1], Broda *et al.* (1995)[2]
cbhII	*P. chrysosporium* ME446 (ATCC 34541)	DNA-PCR	CCTCAGCCCTTACTACGC [#29]	CCAATCTACCTCTACAGC [#32]	Not given[1], 950[2], 1330[2]	Tempelaars *et al.* (1994)[1], Broda *et al.* (1995)[2]
cel3	*Agaricus bisporus* D649	DNA-PCR	T CCTTGATCNACGATAAAATG C G A G G [OL60B1]	GTAATACGACTCACTATAGGGC [T7]	400, 500, 1100	Chow *et al.* (1994)
cbh1-4	*P. chrysosporium* BKM-F 1767 (ATCC 24725)	RT–PCR	GATCGAATTCATGTTCCGCGCCGCCGCACT [CBH-5′]	GATCGAATTCTTAGTAGCACTGCGAGTAGT [CBH-3′]	1537	van Rensburg *et al.* (1996)
cbh1-1	*P. chrysosporium* BKM-F 1767	RT–PCR	TCCTTTTGTTGGGCGTCGTC	ACCTCCAACACGGGCTTTG	766, 8176	Lamar *et al.* (1995)
cbh1-4	*P. chrysosporium* BKM-F 1767	RT–PCR	AAGGTCGTCCTCGACTCGAA	CTCCAAGCCCTTCACCGTCG	741, 7795	Lamar *et al.* (1995)

[a]Superscripts in the 'Amplified product' column refer to the corresponding superscripts in the 'Reference' column.

product hybridized to a 1.4 kbp *cel* mRNA; the 600 bp product to a 1.4 kbp mRNA as well as to a small mRNA thought to be non-*cel* mRNA. The 1.1 kbp PCR-amplified product was assumed to be an almost full-length copy of the *cel3* cDNA. The 400 bp PCR product was digested with *Eco*RI to remove a small terminal vector sequence, labelled with ^{32}P-dCTP, and used as a probe to screen the cDNA library (Chow *et al.*, 1994) to obtain 14 cDNA clones which were then analysed. The nucleotide sequence of the clones showed that two types of cDNAs were isolated. Using genomic DNA from four monokaryotic mycelia, the segregation pattern of the *cel3* gene was studied. Since two differing patterns of hybridization were obtained in Southern hybridization analysis, it was concluded that the two genes were allelic (Chow *et al.*, 1994). These alleles had 98.8% sequence identity to each other.

A cDNA fragment encoding the *P. chrysosporium* cellobiohydrolase (*cbh1-4*) was amplified using RT–PCR and cloned (van Rensburg *et al.*, 1996). First-strand cDNA was synthesized from *P. chrysosporium* BKM-F 1767 poly(A)-RNA using a commercial kit and commercial primers (Boehringer Mannheim). The DNA was then amplified using two primers (Table 10.4), CBH-5′ (forward primer), and CBH-3′ (reverse primer) based on a previously published *cbh1-4* gene sequence (Vanden Wymelenberg *et al.*, 1993). The 1537 bp PCR-amplified product was determined to be identical to the published nucleotide sequence of *cbh1-4*, minus the two introns (Vanden Wymelenberg *et al.*, 1993). It was subcloned into the yeast multicopy episomal plasmid pJC1 by inserting it between the yeast phosphoglycerate kinase-I gene promoter ($PGK1_P$) and terminator ($PGK1_T$) sequences. The recombinant plasmid (pCBH) contained the $PGK1_P$-*cbh1-4*-$PGK1_T$ construct, designated CBH1. A 3477 bp *Pvu*II DNA fragment containing this CBH1 construct was excised from pCBH and subcloned into a unique *Sma*I site of another plasmid, pEND. This plasmid consisted of the yeast multicopy episomal, expression/secretion vector (pDLG4) and the gene *end1*, which encodes the endo-β-1,4-glucanase of *Butyrivibrio fibrisolvens*, a cellulolytic rumen bacterium. The resulting 12 kbp plasmid, designated pENCB, was transformed into the yeast *Saccharomyces cerevisiae*, and the coexpression of *cbh1-4* and *end1* was analysed. The maximum cellulolytic activity of the transformed *S. cerevisiae* Y294 [pENCB], containing both the genes, was 1380 U as compared to *S. cerevisiae* with *cbh1-4* (which had a maximum cellobiohydrolase activity of 12.03 U) and *end1* (which had a maximum endo-β-1,4-glucanase activity of 1100 U). These studies indicated that coexpression of both the genes results in enhanced cellulose degradation (van Rensburg *et al.*, 1996).

Cellobiose dehydrogenase (CDH) is a haemoflavoenzyme, secreted under cellulolytic conditions, that oxidizes cellodextrins, lactose and mannodextrins (Eriksson *et al.*, 1990). The role of CDH in wood degradation, and catabolism of cellobiose has been suggested (Bao *et al.*, 1993; Eriksson *et al.*, 1993; Ander, 1994). In a recent study, Raices *et al.* (1995) isolated more

than 50 putative positive clones, using either immuno- or gene-probe screening for the CDH gene. Out of these clones, five were sequenced and found to overlap with identical sequences, but lacking the 5′-end of the mRNA. This 5′-end was obtained by RACE (Frohman *et al.*, 1988), using poly(A)-RNA as the template and two nested primers (sequences not shown) based on the 5′-end of the longest cDNA isolated. A single PCR product, Ra-12, corresponding to the missing 5′-end, was obtained. By determining the sequences of the 5′ (Ra-12) and 3′ (CP3) sections of the CDH gene, the complete cDNA sequence was determined (Raices *et al.*, 1995). The full-length cDNA was assembled by combining Ra-12 and CP3 in a recombination PCR using the Ra-12 forward and the CP3 reverse primers (sequences not shown). The complete cDNA contains 2310 translated bases excluding the 3′ poly(A)-tail. Analysis of the CDH amino acid sequence showed the presence of haem and flavin adenine dinucleotide (FAD) domains, as well as the nucleotide-binding motif. The FAD domain appears to be distantly related to the glucose/methanol/choline (GMC) oxidoreductase family (Raices *et al.*, 1995).

Xylanase genes

Graham *et al.* (1993) used a novel PCR-based procedure to synthesize an artificial xylanase gene from the white-rot fungus *Schizophyllum commune*. The sequence of the synthetic gene was designed so that restriction enzyme recognition sequences could be introduced into the gene without alteration of the amino acid sequence of the final translation product. Additionally, the design also incorporated a selection of codon bias suited for expression in *E. coli*. Overlapping PCR primers Xyl1 (189-mer) and Xyl2 (208-mer) were mixed with different dilutions of two other primers, Xyl3 (42-mer) and Xyl4 (36-mer), in a PCR-amplification procedure to yield a 426 bp double-stranded product XylA, representing two-thirds of the 5′-end of the xylanase gene. An aliquot of XylA was mixed with a 217-mer oligonucleotide primer and flanking primers Xyl3 (5′-end) and Xyl6″ (3′-end) and subjected to a second PCR to yield a 629 bp double-stranded XylB DNA representing the full-length xylanase-encoding *xynA* gene (Graham *et al.*, 1993). The XylB fragment was subcloned into a plasmid vector; after sequencing three subclones, it was found that errors were introduced in the sequences of each of the subclones because of the low fidelity of *Taq* polymerase. The error was corrected by selecting one of the subclones and excising the segment containing the error using flanking restriction sites and replacing it with a correct segment from another subclone; the result was an authentic *xynA* gene. The synthesized *xynA* gene was subcloned into the *Nhe*I–*Hin*dIII site of the protein expression vector, pTUG. To accomplish this, the 5′ *Pst*I site of *xynA* was changed to a *Nhe*I site by PCR amplification using a new 5′ primer Xyl3″ (with a *Nhe*I recognition site) and the original 3′ primer Xyl6″. The PCR product was then subcloned into pTUG and, after verification that the sequence was correct, was transformed into *E. coli* JM101. Xylanase-

secreting clones produced clearing zones when grown on Luria broth agar plates containing remazol brilliant blue xylan substrate.

Using the data obtained from the N-terminal sequence of purified xylanase APX-II from the xylanolytic fungus *Aureobasidium pullulans* Y-2311-1, Li and Ljungdahl (1996) designed two degenerate biotinylated oligonucleotide primers, P0813 and P3035 (Table 10.5) to amplify a xylanase gene from genomic DNA. The resulting 83 bp biotinylated PCR fragments were used as probes to screen a cDNA library, as well as in the characterization of genomic DNA (Southern blot), and in the study of regulation of the xylanase gene (Northern blot). Five positive clones were obtained after screening the cDNA library of which two were sequenced. The sequence data revealed that none of these clones contained the 5′-end of the gene. Using PCR, the 5′-end of the cDNA was amplified from the cDNA library, using two oligonucleotide primers. The forward primer (T3 promoter sequence; P200) was located at the 5′ of the insert while the reverse primer (P3338) was 152 bp from the 5′-end of the positive cDNA clones (Table 10.5). The PCR product was cloned and sequenced and using the sequence data, two oligonucleotide primers PFW and PRW (Table 10.5) were synthesized. PFW and PRW corresponded to the 5′- and 3′-ends of the full-length cDNA for the *A. pullulans* xylanase, and were used to amplify the whole gene. Comparison between the genomic and cDNA sequences revealed that one intron of 59 bp was present in the coding region. The result of the Southern blot showing the presence of only a single copy in the genome, and that of the Northern blot showing that only a single transcript was present, indicated that the two xylanases present in the culture were encoded by the same gene (Li and Ljungdahl, 1996).

10.4.2 Allele-specific oligonucleotides

The PCR approach was also used to differentiate *lip* alleles of *P. chrysosporium* (Gaskell *et al.*, 1992, 1994, 1995). Two primers, A and B (Table 10.1), were used to amplify *lip* H8 sequences from the genomic DNA of basidiospores (homokaryotic) and dikaryote isolates of *P. chrysosporium*. Amplified products of 251 bp and 260 bp were obtained. These PCR products were Southern blotted and probed with gene-specific oligonucleotide probes to identify rapidly the H8 gene and its alleles (Gaskell *et al.*, 1992).

In another study, Gaskell *et al.* (1994) developed a strategy for genetic mapping by segregation analysis. They monitored allelic segregation by PCR of genomic DNA from single basidiospore-derived cultures followed by probing with allele-specific oligonucleotides. Gene-specific PCR primers were used for 12 *P. chrysosporium* genes coding for LIP (Table 10.1), glyoxal oxidase (GLOX) and CBH (Table 10.4). Based on the sequences of the amplified products, allele-differentiating probes were prepared. Genetic linkage was determined by monitoring the segregation of specific alleles

Table 10.5. Primers used for PCR amplification of xylanese-encoding genomic and cDNA sequences.

Gene	Organism	PCR procedure	5′ Primer	3′ Primer	Amplified product (bp)	Reference
Xylanese [*xylA* = 5′-end of *xynA*]	*Schizophyllum commune*	DNA-PCR	ACCCCGTCTTCTACCGGTACCGACGGTGGTTA-CTACTACAGCTGGTGGACCGACGGTGCTGGTG-ACGCTACCTACCAGAACAACGGTGGTGGTTCT-TACACGTTAACCTGGTCTGGTAACAACGGTAA-CTTAGTTGGTGGTAAAGGTTGGAACCCGGGTG-CTGCTTCTAGATCTATCTCTTACTCTGGT [Xyl1] (189-mer) ACCGCCTCTGCAGCTTCTGGTACCCCGTCTTC-TACCGGTAC [Xyl3] (42-mer)	GACGGAGCGTTGTAACGCCAGGTAGACAGGA-TGTCGTAGGTGGCGCCGTTGCAGGTAACGGA-TCCTTTGTGAGAAGCAGCAGAAGACGGGTCG-TAAGAACCGTAAGATTCAACGATGTAGTATT-CGATCAGACTCGAGCGGGTCCAACCGTAAAC-AGACAGGTAAGAGTTACCGTTCGGCTGGTAG-GTACCAGAGTAAGAGATAGATC [Xyl2] (208-mer) CTGGGTACCATCGATAGACGGAGCGTTGTAA-CGCCA [Xyl4] (36-mer)	426	Graham *et al.* (1993)
Xylanase [*xylB* = *xynA*]	*Schizophyllum commune*	DNA-PCR	ACCGCCTCTGCAGCTTCTGGTACCCCGTCTTC-TACCGGTAC [Xyl3] (42-mer)	CTAGACGGTGATGGTAGCGGTACCAGAAGAC-TGGTAACCTTCGGTCGCGACGATCTGGTAGT-TGTGTTCAGAACCCAGGTTCATACCCAGGCC-TTTCCAAGCGTCGAAGTGGCACTGAACGTCG-ACGGTACCAGAGATAGAACCACCCGGAGCTT-TTTTCGGGTTACGTACAGACCAGAACTGTTC-GAAGGTCTGGGTACCATCGATAGACGGAGCG-TTGTA [Xyl5] (222-mer) CGTATAAGCTTCTATTAGCTAGCGGTGACGG-TGATGGTAGCCG [Xyl6″] (43-mer)	629	Graham *et al.* (1993)

Xylanese [*xynA*]	*Schizophyllum commune*	DNA-PCR	GCCTCTGCTAGCGGTACCCCGTCTTCTACCGG-TAC [Xyl3″] (35-mer)	CGTATAAGCTTCTATTAGCTAGCGGTGACGG-TGATGGTAGCCG [Xyl6″] (43-mer)	Not given	Graham *et al.* (1993)
Xylanese [3′-end of *xynA*]	*Aureobasidium pullulans* Y-2311-1	DNA-PCR	TATGTNCAAAATTATAA C G C C [P0813]	CCATTATTCCAATACAT G G G [P3035]	83	Li and Ljungdahl (1994)
Xylanese [5′-end of *xynA*]	*Aureobasidium pullulans* Y-2311-1		GTCGCCATTGACACCGT [P200]	GAAGTCGCCATTGACACCGTTGTT [P3338]	Not given	Li and Ljungdahl (1994)
Xylanese [*xynA*]	*Aureobasidium pullulans* Y-2311-1	DNA-PCR	CGGCACGAGCTCGTGCCGG [PFW]	GTAGCAAGGTGTCTGACAT [PRW]	895	Li and Ljungdahl (1994)

among homokaryotic segregants. Homokaryotic single-basidiospore cultures were first isolated and identified, followed by the extraction of genomic DNA, amplification of genes, and differentiation of alleles using radiolabelled oligonucleotides as probes. The linkage was computed from allelic co-segregation frequencies. Five linkage groups were identified, one of which contained eight closely linked *lip* genes. One unlinked *lip* gene, was determined to co-segregate with a *cbh1* gene cluster (Gaskell *et al.*, 1994).

Recently, Gaskell *et al.* (1995) investigated the copy number, inheritance, genomic location, and distribution of Pce1, an insertional element within *P. chrysosporium* gene *lipI*, discovered after analysis of the sequences of the ten known *lip* genes. PCR was used to amplify a 1767 bp Pce1 insertional element from genomic DNA of *P. chrysosporium* BKM-F 1767, using primers at the junction of the insertion point of Pce1 in *lipI2*. The Pce1 element was localized to the 3.7 Mb chromosomal band by experiments using the amplified product to probe Southern blots of clamped homogeneous field electrophoresis gels of size-fractionated chromosomes of a dikaryotic strain of *P. chrysosporium* BKM-F 1767. The copy number and distribution of Pce1 were assessed by Southern blot analysis of restriction enzyme-digested genomic DNA. An additional Pce1-like sequence was found on a 4.6 kbp *Bam*HI–*Xba*I genomic DNA fragment. Southern blot analysis of genomic DNA from 17 strains of *P. chrysosporium* using Pce1 as a probe showed that only three isolates hybridized under moderate stringencies. One widely used laboratory strain, ME-446, did not show hybridization with Pce1. Allelic segregation patterns were determined for *lipA* and *lipI* using PCR with gene-specific primers and 69 single-basidiospore isolates. PCR products of 2236 bp and 479 bp allowed easy differentiation of *lipI* alleles. For *lipA*, allele-specific probes were used to differentiate PCR products, since all the *lipA* gene-specific PCR products were identical in length. The results showed that *lipA* and *lipI* alleles co-segregate. RT–PCR followed by probing with allele-specific oligonucleotides, was also done in order to detect *lipI1* and *lipI2* transcripts in N-limited and C-limited cultures (discussed in section 10.4.3).

10.4.3 Gene expression under varying environmental conditions

Stewart *et al.* (1992) used competitive RT–PCR and gene-specific oligonucleotide probes to study the pattern of LIP gene expression in *P. chrysosporium* BKM-F 1767. First-strand cDNA of four closely related LIP genes (*lipA*, *lipB*, *GLG5* and *0282*) was prepared by reverse transcription using a conserved downstream primer (Table 10.1). Gene-specific upstream primers were used to amplify the double-stranded cDNA. Each PCR contained genomic DNA as a competitive template. The cDNA levels of the four genes were quantified in N-limiting and C-limiting culture conditions. The transcript levels for the LIP H8-encoding *lipA* and *lipB* genes, and for the

LIP H2-encoding *0282* gene, were similar in C- and N-limited cultures. However, for the LIP H10-encoding *GLG5* the N-limited cDNA levels were between 25 and 100 pg, but no cDNA product was amplified in the C-limited cultures. Competitive PCR was also used to quantify the LIP H2-encoding *GLG4* and *V4* transcript levels, using gene-specific primers (Table 10.1). The *GLG4* transcript was more abundant (1000-fold) in C-limited than in N-limited cultures. The *V4* gene transcript could only be detected using a second set of primers (Table 10.1) and when reverse transcription reactions included 0.5 μg of RNA and were extended to 45 min. Under these conditions, the *V4* gene transcripts were found to be more abundant in N-limited than in C-limited cultures.

Brooks *et al.* (1993) determined the expression profiles of LIP H8-encoding *LIG1* and LIP H2-encoding *LIG5* in *P. chrysosporium* ME-446 cultures under conditions of nitrogen limitation (LN) or non-limitation (HN) using gene-specific primer pairs (Table 10.1). No *LIG1* expression was seen in either LN or HN cultures, but *LIG5* expression was seen in both LN and HN cultures. In carbon non-limiting cultures, however, no *LIG5* transcripts were seen, leading to the conclusion that *LIG5* expression in strain ME-446 is dependent on low C concentration.

The expression of LIP genes from C-limited cultures of *P. chrysosporium* BKM-F 1767 was analysed by PCR. For this, cDNA prepared from 6-day-old C-limited cultures was used as the template with degenerate oligonucleotide primers corresponding to the regions surrounding the proximal and distal His residues in LIP from BKM-F 1767 (Reiser *et al.*, 1993). Twenty-four PCR clones were analysed and found to be ordered into two sets, based on digestion with the restriction enzymes *Sal*I and *Rsa* I. One set showed a high degree of similarity to the LIP H2-encoding *CLG4* cDNA sequence of de Boer *et al.* (1987), while the other was similar to the LIP H8-encoding *L18* cDNA sequence (Ritch *et al.*, 1991). These represented the major transcripts in C-limited cultures.

Broda *et al.* (1995) applied the RT–PCR technique to monitor the expression of 11 genes from low-N cultures of *P. chrysosporium* ME-446 during growth with one of four different carbon sources: low glucose (0.2%), high glucose (2.0%), Avicel (0.2%) and ball-milled straw (BMS; 0.2%). Different primers were used for amplification of LIP H8-encoding genes *LIG1*, *LIG2*, *LIG3*, and *LIG4*, the LIP H2-encoding gene *LIG5*, the unknown LIP-encoding gene *LIG6* (Table 10.1), the MNP-encoding gene *mnp1* (Table 10.2), and the CBH-encoding genes *cbh1.1*, *cbh1.2*, and *cbhII* (Table 10.4). Amplification of the constitutively expressed *trpC* gene was used as a positive control for gene expression in all cultures (primers for *trpC* are shown in Table 10.6). Cultures grown in low glucose expressed only *LIG5* (on days 3–8), and *cbh1.1* (on days 7 and 8). High glucose cultures expressed none of the genes studied, except low levels of *mnp1* on day 3. Avicel cultures expressed *cbh1.1* and *cbh1.2* on days 3–6; *LIG1* and *LIG2* on day 6, *LIG3* on days 4–8, *LIG6* on day 3, and *cbhII*, *LIG5* and *mnp1* on days

Table 10.6. Primers used for PCR amplification of genomic DNA and cDNA of house-keeping and other genes isolated from ligninolytic fungi.

Gene	Organism	PCR procedure	5′ Primer	3′ Primer	Amplified product (bp)	Reference
ITS[a]	*P. chrysosporium* BKM-F 1767, *P. chrysosporium* HHB-6251-sp, *P. chrysosporium* FP-104297-sp, *P. sordida* HHB-7423-sp, *P. sordida* HHB-8922-sp, *F. fomentarius, Penicillium sp., Pythium sp., P. radiata, R. solani*	DNA-PCR	GGAAGTAAAAGTCGTAACAAGG [ITS 5]	TCCTCCGCTTATTGATATGC [ITS 4]	700	Johnston and Aust (1994b)
β-tubulin	*P. chrysosporium* BKM-F 1767 (ATCC 24725)	RT–PCR	AGGTCGTCTCAGACGAACAC	GGCATGGGTACTCTCCTGATC	495, 640	Lamar *et al.* (1995)
trpC	*P. chrysosporium* ME446 (ATCC 34541)	RT–PCR	CACGGGCATCGTGACGGATAC	TGGGTCTTGAGTGTGTAGTGG	126, 179	Broda *et al.* (1995)
Pcel	*P. chrysosporium* BKM-F 1767 (ATCC 24725)	DNA-PCR	GGGACCGTAGCTACATCAGCAAT	CTGGCTGGGTACATCAG	1747	Gaskell *et al.* (1995)

[a]Presented here as an example of similar papers published using the internal transcribed spacer (ITS) region or other regions of fungal ribosomal DNA for phylogenetic studies of lignocellulosic fungi.

3–8. BMS cultures expressed *cbh1.1* and *cbh1.2* on days 3–6, *LIG1* on day 8 and *cbhII*, *LIG5* and *mnp1* on days 3–8.

RT–PCR and allele-specific oligonucleotide probes were used to detect the LIP H2-encoding *lipI* transcripts in N-limited and C-limited cultures of *P. chrysosporium* BKM-F 1767 (Gaskell *et al.*, 1995) using gene-specific primers (Table 10.1). The fungus expressed *lipI1* transcripts in both culture media, but *lipI2* transcripts were not detected; the *lipI2* contains a 1747 bp insertional element Pce1 (described in Section 10.4.2). The results indicated that Pce1 inactivated the *lipI2* gene.

Rajakumar *et al.* (1996) used RT–PCR to determine whether LIP-like genes were transcribed in *P. sordida* and *C. subvermispora* cultures grown under ligninolytic (N-limited medium) conditions. The primers used are shown in Table 10.1. The results showed that one LIP-like sequence, with a 88.4% nucleotide similarity to the corresponding region of a *T. versicolor lip* gene, was transcribed in *P. sordida* cultures. However, no LIP-like transcripts were detected in *C. subvermispora* cultures.

Quantitative RT–PCR analysis was done on mRNA isolated from anthracene-transforming soil cultures of *P. chrysosporium* BKM-F 1767 to assess the temporal regulation of the H_2O_2-producing glyoxal oxidase (GLOX) gene expression (Bogan *et al.*, 1996b). Anthracene, a known LIP substrate, needs the presence of H_2O_2 for its transformation by LIP. The primers used for PCR amplification of the *lip* gene sequences are shown in Table 10. 1; no primer sequences for *glx* were given. The results showed that during the initial period of incubation (<10 days), the LIP H8-encoding *lipA* and the LIP H2-encoding *lipD* transcripts were at the highest levels. The highest transcript level for any of the genes studied was that of the LIP H2-encoding *lipJ* (days 15–20). The *lipA* transcript level, however, maintained high levels for a majority of the 25 days. No *lipF* (unknown LIP-encoding) transcripts were seen on any of the days studied. The transcript levels of *lipA* and *glx* genes correlated with the levels of the enzymes they coded for (H8 and GLOX, respectively). The oxidation of anthracene was also seen to take place throughout the course of the study (Bogan *et al.*, 1996b).

In a separate study, Bogan *et al.* (1996a) used competitive RT–PCR to examine the temporal expression of three MNP genes (*mnp-1*, *mnp-2* and *mnp-3*) of *P. chrysosporium* BKM-F 1767 during the bioremediation of PAH-contaminated soil. All three transcripts were seen on day 1 or 2, with peak levels on day 6. The β-tubulin gene (*tub*) and glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) transcripts were also determined by competitive RT–PCR as controls for mRNA loading. The results showed that *mnp* mRNA levels correlated with MNP enzyme levels and with the disappearance of PAHs from the soil, supporting the hypothesis that MNP are involved in the oxidation of PAHs in soil (Bogan *et al.*, 1996a).

Competitive RT–PCR was used to determine the relative transcript levels of *P. chrysosporium* BKM-F 1767 *cbh1-1*, *cbh1-2* and *cbh1-3* genes in

glucose-grown, sucrose-grown and cellulose–cellobiose-grown cultures (Covert *et al.*, 1992). First-strand cDNAs were prepared by reverse transcription with downstream primers (see Table 10.4). This was followed by PCR-amplification using upstream primers (Table 10.4) in the presence of serial dilutions of genomic DNA as competitive templates. The primers flanked an intron common to all three genes, so that the PCR-amplified products obtained from cDNA and genomic DNA could be identified on agarose gels. Gene-specific oligonucleotide probes were used to further establish the identity between the three genes. The results showed that *cbh1-3* transcripts were about 1000-fold more abundant than those of *cbh1-1* and *cbh1-2* in cellulose–cellobiose-grown cultures. The *cbh1-1* and *cbh1-2* transcript levels were low in all four cultures, but were higher than *cbh1-3* transcript levels in 3-day-old glucose-grown cultures. Thus, unlike *cbh1-3*, *cbh1-1* and *cbh1-2* appear not to be subjected to glucose repression.

10.5 Future Perspectives

An increasing number of researchers have been using PCR methodology in recent years for studies on fungal lignocellulose degradation. The techniques applied to date have been limited to DNA-PCR, RT–PCR, competitive RT–PCR and inverse PCR. These techniques have been used for the detection, isolation and characterization of genes involved in lignocellulose degradation. The latter included genes encoding LIP, MNP, laccase, cellulase and xylanase. PCR approaches have also played a major role in the study of regulation of expression of genes encoding lignocellulose-degrading enzymes under a variety of environmental conditions. Furthermore, PCR techniques were also useful for mapping and segregation analysis of genes involved in lignin and cellulose breakdown as well as for studying the molecular genetics of an insertional element Pce1 in *P. chrysosporium*.

The PCR approaches described above have been useful in enhancing our understanding of fungal lignocellulose degradation but these studies represent only the beginning of what we believe will be an expanding field in future research. Several new and improved PCR-based techniques show promise for future applications in this research area. For example, DDRT–PCR (Liang and Pardee, 1992; see Chapter 3) is a sensitive technique that could be used for detailed studies on environmental control of gene expression. Another area where PCR could be used effectively would be in phylogenetic studies of lignocellulose-degrading wood-rot basidiomycetes (Hibbett and Vilgalys, 1991; Kwan *et al.*, 1992; Boysen *et al.*, 1996; Bunyard *et al.*, 1996; Chiu *et al.*, 1996). More recently, in the Center for Microbial Ecology at Michigan State University, we have initiated studies using denaturing gradient gel electrophoresis PCR (DDGE–PCR) to characterize the phylogenetic affiliation of the various lignin-degrading soil basidiomycetes. Thus it appears quite likely that a greater variety of PCR approaches

will be used by researchers in the future for increasing the knowledge of fungal lignocellulose degradation systems.

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