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Bioremediation - An alternate utility of cloned lignin peroxidase from *Phanerochaete chrysosporium* *

C LOGESWARI MONICA[1], K G TIRUMURUGAAN[2], K VIJAYARANI[3], C CHANDRASEKARAN[4] and K KUMANAN[5]

Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu 600 051 India

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ABSTRACT

Physical, chemical and biological methods were explored to identify a practicable solution for bioremediation. Biological methods always have remained attractive and in this context we targeted *Phanerochaete chrysosporium* a wood degrading fungi that is not only well known for its degradation ability but also a producer of lignin modifying enzymes. This fungi was grown under nitrogen limiting conditions and the peak ligninase activity could be observed on the fourth day in the culture supernatant as detected by methylene blue and veratryl alcohol assay. The full-length gene of the major isoform of lignin peroxidase (LiP) the LiP-H8 was amplified and cloned into pYES-DEST52 expression vector. The active expressible LiP-H8 expression clone was confirmed by employing an agar plate incorporated with methylene blue which resulted in a zone of clearance after 48 h indicating its degradation capability. Further studies by transformation of the confirmed LiP-H8 clone into laboratory strains of *Saccharomyces cerevisiae* and assessing the enzyme activity would determine the future implications of this recombinant lignin peroxidase.

Key words: Azo dyes, Bio-degradation, Gel assay, Lignin peroxidase, White rot

India being the 12th largest economy of the world has the largest population of livestock (20%) which contributes to around 24% of agricultural GDP. Under Indian conditions, ruminants are mainly fed on low quality roughages that are poorly digested in the rumen leading to low production efficiency. Research has shown that greater increase in animal productivity and efficiency could be achieved with subtle changes in the balance of nutrients in the feed base (Leng 1990) to meet the future demands. The complex nature of the matrix of the roughages is due to the 3 basic polymers such as cellulose, hemicellulose and lignin. The lignin being the second most abundant plant polymer needs to be opened in the roughages to expose the cellulose and hemicellulose matrix for enzyme digestion. In spite of various approaches available for efficient breakdown of the plant polysaccharide matrix, lignin degrading enzymes have been shown to very much efficient in addition to the battery of endo- and exo-acting glycosyl hydrolases (Jung and Allen 1995). On the other hand the increased industrialization effort that is being made in our country to target development in economic terms has been contributing a major disturbance to the environmental balance. Among the toxic and hazardous industrial pollutants, the synthetic dyes (mostly azo dyes) and their intermediates which are commonly used in textile industries are of a major concern. An estimate indicated that almost 10–15% (280 000 tonnes) of the dye used in manufacturing and textile industry is released into the environment worldwide (Mass and Chaudhari 2005) leading to pollution of the water resources. Synthetic dyes are not biodegradable and when discharged into the environment, these are persistent and many are also toxic (Pointing 2001).

Several physical, chemical and biological methods were explored to identify a panacea applicable to these 2 major areas. Biological methods employing different naturally occurring organisms that efficiently degrade plant biomass or synthetic dyes have been a major research topic in many of the laboratories. *Phanerochaete chrysosporium,* a well known white rot fungi that belongs to the family *Basidiomycotina,* are very well known for their specialized degradation abilities (Martinez *et al.* 2004) as they excrete 3 valuable and chemically distinct extra cellular enzymes; referred to as lignin-modifying enzymes (LMEs), which are critical to open the complex three dimensional structure of

*M. Phil thesis work of the first author.

Present address: [2]Department of Animal Biotechnology (drthiru20@yahoo.com, tirumurugaankg@tanuvas.org.in), Madras Veterinary College. [5]Director of Research (monimails @yahoo.co.in, tirumurugaankg@tanuvas.org.in, ranikumanan65 @hotmail.com, chandruz_ibt08@yahoo.com, kumananrani @hotmail.com).

lignin to be used in metabolism during nutrient starvation (Kirk *et al*. 1986, Gold and Alic 1993, Akin *et al*. 1995). Two of them are glycosylated heme-containing peroxidases [lignin peroxidase (LiP; E.C. 1.11.1.14) and Mn dependant peroxidase (MnP; E.C.1.11.1.13)] and the third one is a copper containing phenoloxidase [laccase (Lac; EC 1.10.3.2)]. The MnP enzyme catalyses an H_2O_2 dependant oxidation of Mn^{2+} to Mn^{3+} that oxidize phenolic components of lignin (Wariishi *et al*. 1992). Degradation of lignin, cellulose, and hemicellulose by white-rot fungi has been shown to be almost at equal rates. Owing to their efficiency in biodegrading natural lignocellulosic materials and humic substances to CO_2 and H_2O, this fungi or its products have the ability to act on a wide variety of environmental organopollutants including pesticides, munitions waste, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, wood preservatives, synthetic azo dyes, and waste materials from paper producing plants and transform and mineralize them *in vitro* or *in vivo* (Hawari *et al*. 1999, Limura *et al*. 1996, Lin *et al*. 1990, Pointing 2001). In carbon or nitrogen starved cultures, *P. chrysosporium* produces several isozymes of LiPs (Leisola and Fiechter 1985, Kirk *et al*. 1986, Renganathan *et al*. 1986, Leisola *et al*. 1987). LiPs exist in at least 10 isoforms with pI values ranging from 3.2 to 4.7 and molecular weights of 38–46 kDa (Renganathan *et al*. 1986, Leisola *et al*. 1987, Kirk and Farrell 1987). Hence, in this study the fungi was grown under *in vitro* conditions suitable for production of LiP, the full-length gene cloned (Lip-H8 the major isoform), expressed in a prokaryotic host and an active expressible clone was confirmed by its ability to degrade an azo dye *in vitro*.

MATERIALS AND METHODS

Microorganism and culture: Phanerochaete chrysosporium was obtained as an active slant from Microbial Type Culture Collection, Institute of Microbial Technology, and Chandigarh, India. These were grown on Sabouraud's dextrose agar (SDA) plates with 2% malt extract and incubated for 4–6 days at 30°C. The fungus from the actively proliferating area were removed, placed in distilled water and stored at 4°C (Burdsall and Dorworth 1994). For RNA extraction and for assessing the lignin peroxidase activity, the fungus was grown in Sabouraud's dextrose broth (SDB) under nitrogen limiting conditions (24mM ammonium tartrate and 0.4mM veratryl alcohol).

Lignin peroxidase activity: The enzyme activity in the culture supernatants was assessed by methylene blue assay (Magalhaes *et al.* 1996) and veratryl alcohol assay (Tien and Kirk 1984). Briefly the culture supernatant (2.2ml) was mixed with 0.1ml of 1.2mM methylene blue and 0.6ml of 0.5M sodium tartrate buffer, the reaction started with the addition of 0.1ml of the 2.7mM H_2O_2 and the decrease in the absorbance was measured at 664nm. For the veratryl alcohol assay 1.8ml of the clarified culture supernatant was mixed with 0.1ml of 50mM veratryl alcohol and 0.5ml of 0.5M sodium tartrate buffer, the reaction was started by the addition of 0.1ml of the 10mM H_2O_2 and the rate of oxidation of veratryl alcohol to veratraldehyde was detected by measuring the absorbance at 310nm.

PCR amplification and cloning of the full-length Lip-H8 gene in to expression vector: The fungal mat grown under nitrogen limiting conditions were harvested on day 4 (peak enzyme activity), the total RNA extracted using reagent following the manufacturer's instructions, RNA was cleaned up using the RNeasy mini kit and the cDNA synthesized using First-Strand synthesis supermix with oligo dT primer. The full-length Lip-H8 (1.12kb) was amplified with DNA polymerase employing LiP-FC 5'CACCATGGCCTTCA AGCAGCTCT 3' and LiP-RC 5'AGCACCCGGAGGCG GAGGGATGC 3' primer pairs under the following conditions: 94°C for 3 min. followed by 1 cycle of 94°C for 1 min, 50°C for 1 min, 68°C for 5 min and 39 cycles of 94°C for 1 min, 56.2°C for 1 min 30 sec, 68°C for 2 min with the final hold at 4°C. The purified PCR amplicons were directionally cloned (due to the presence of CACC in the forward primer) into the pENTR-D TOPO gateway entry cloning vector and the kanamycin resistant recombinant colonies were confirmed by RE digestion and sequencing. *In-vitro* recombination of the pENTR-D-Lip-H8 plasmid and destination vector was performed using clonase enzyme mix to obtain the recombinant expression vector (pYES-LiP). The pYES-LiP with the full-length Lip-H8 gene was confirmed by colony PCR using the vector and insert reverse primer on ampicillin resistant colonies, restriction enzyme digestion (*KpnI)* and also by sequencing.

Ingel- enzyme assay for expression of the recombinant lignin peroxidase: The ability of the pYES-LiP expression vector to produce lignin peroxidase was assessed by in-gel dye degradation assay (Borokhov and Rothenburger 2000). One per cent stock solution of the methylene blue dye was filter sterilized and added to autoclaved LB agar to a final concentration of 0.01% and wells were punched in the agar after solidification. From an overnight culture of pYES-LiP vector in LB broth with ampicillin, 50 µl was added to each of the wells and incubated for 48 h. Hydrogen peroxide (2.7mM) was added to initiate the reaction and expression of lignin peroxidase by the pYES-LiP vector was confirmed by the zone of clearance (degradation of the methylene blue).

RESULTS AND DISCUSSION

Among the naturally occurring organisms the white rot fungi (*P.chrysosporium*) is very well known for its lignin degrading enzymes with redox potential beyond the reach of other peroxidases, along with the battery of other exo-and endo-glycosyl hydrolases (Kirk and Cullen 1998). We report herewith the cloning and successful expression of the major isoform of lignin peroxidase from *P.chrysosporium* and demonstrate its ability to degrade an azo dye *in-vitro.*

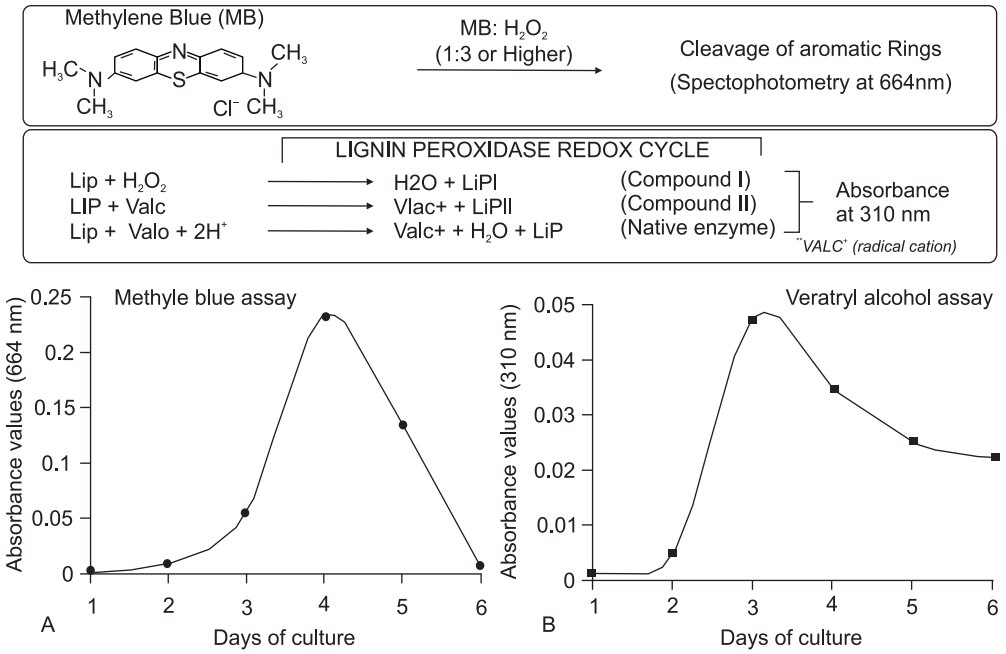

Fig. 1. Determination of lignin peroxidase activity in Sabouraud's dextrose broth with nitrogen limiting conditions inoculated with *Phanerochaete chrysosporium.* **A.** Estimation of the LiP activity in the culture media by methylene blue assay. Note: The enzyme activity could be detected as early as 2 days following culture and peak activity observed on fourth day of the culture. (The reaction details shown in the insert). **B.** Estimation of the LiP activity in the culture media by veratryl alcohol assay. Note: The enzyme activity could be detected as early as 3 days following culture and peak activity observed on fourth day of the culture. (The reaction details shown in the insert.)

Lignin peroxidase existed in several isozymic forms (LiPH1– LiPH10) and the balance between the different isoforms depends not only on the strain, but also on the growth condition (Kirk *et al.* 1986, Leisola *et al.* 1987). The role of individual isozymes in lignin degradation and their underlined genetic regulations are poorly understood. The H8 isoform of LiP was showed to be the predominant ligninase (Tien 1987). Several procedures have been described for growth and maintenance of *P.chrysosporium* for ligninase production (Tein and Kirk 1988, Deepak and Chen 2008) and supplementation of veratryl alcohol has been found to be very efficient which also acts as a stabilizer (Leisola *et al*. 1984, Kirk *et al*. 1986, Cancel *et al*. 1993). The experimental conditions utilizing nitrogen limiting medium in this study resulted in the enzyme production that could be detected in the culture supernatants as early as second day in methylene blue assay but third day in veratryl alcohol assay. The activity of the enzyme peaked on day 4 as detected by both the methods (Fig.1).

Based on the enzyme assay results the fungal mat was collected from the liquid culture on day 4 for RNA extraction, cDNA synthesis, amplification and cloning of full length LiP gene. Our initial attempts with the fungal RNA kit or Trizol method, RT-PCR, sequencing and BLAST analysis showed similarity to complete coding sequences (including the intronic region) indicating DNA contamination in the extracted RNA sample (data not shown). Clean up of extracted total RNA with column based secondary purification that was found to be useful not only in removing DNA contamination but also effectively removes traces of RNases (Mathews *et al*. 2002). Hence, we cleaned up the TriZol extracted RNA using RNeasy mini kit that resulted in not only a very good quality RNA as evidence by the 260/280 ratio of more than 2 by spectrophotometery. RT-PCR could amplify a product of around 1.1 kb (Fig. 2A) which was purified and sequenced with overlapping primers. The assembled sequence on blast analysis showed 100% similarity to the full length major isoform namely the LiP-H8 mRNA (M27401). The sequence results also indicated that the amplified product included the 28 amino acid presumed signal peptide (1–21 amino acids) and pro-peptide sequence (22–28 amino acid) as shown in the previous reports (Schalch *et al*. 1989, Doyle and smith 1996).

The benefits and use of lignolytic enzymes has been very well appreciated due to its role in bioremedial waste treatment and also in catalyzing different chemical transformations. Lignin peroxidase also finds enormous application in agricultural and environmental disciplines due to their role in lignin biodegradation (Kirk and Farrell 1987). The method that early studies utilized for the purification of the active enzyme from *P.chrysosporium* involved purification from native fungus (Tien and Kirk 1984), genetically engineered *P.chrysosporium* (Randall *et al.* 1989, Alic *et al.* 1990) and heterologous expression (Jhonson and Li 1991, Doyle and smith 1996, Nie *et al.* 1998). Expression of LiP in yeast strains could enable *in-vitro* production of this enzyme with many

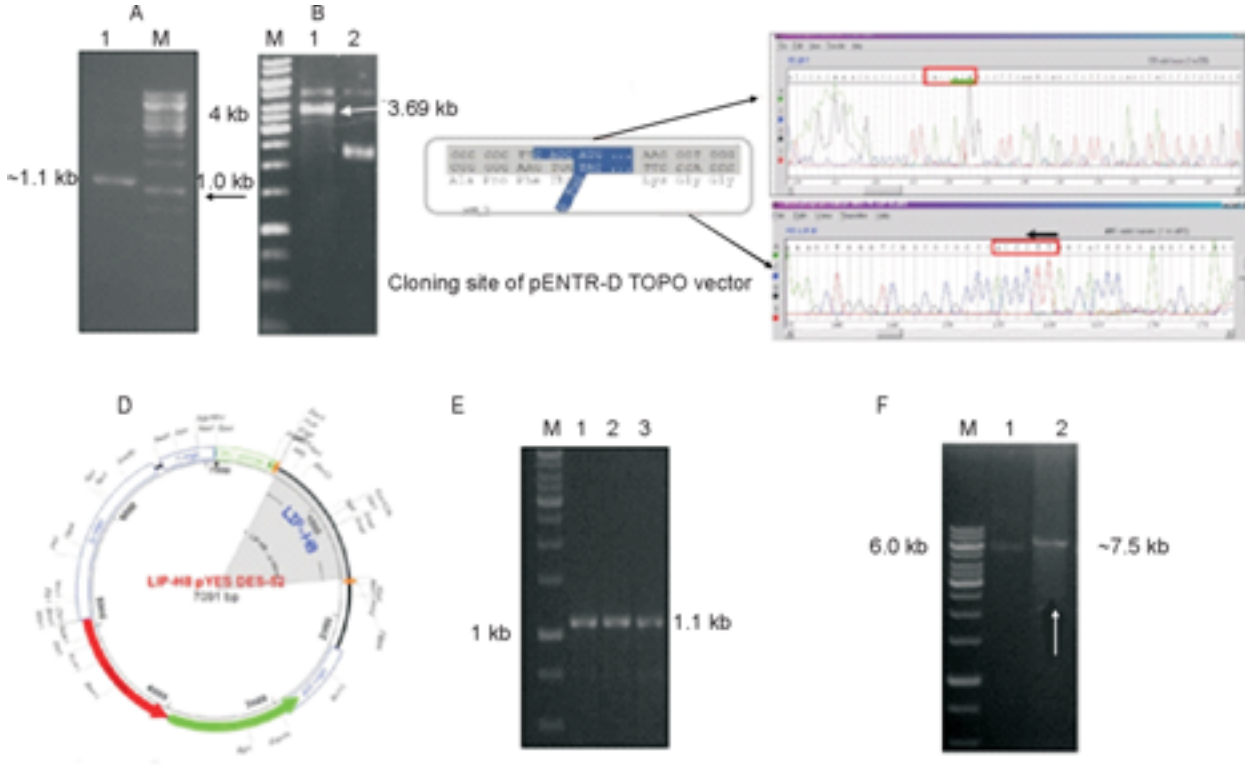

Fig. 2. Reverse transcription PCR amplification and cloning of the full-length Lip-H8 gene in to pYES-DEST 52 expression vector. **A.** RT-PCR amplicon of the full-length LiP gene generated with the LiP FC/RC; **B.** RE digestion of the Lip-H8 entry clone; Lane 1- Recombinant pENTR-Lip-H8 plasmid vector linearized with KpnI (RE site in the LiP-H8 gene around ~3.69 kb); Lane 2 – Uncut plasmid; **C**. Sequencing of the LiP-H8 gateway entry clone to confirm the orientation and open reading frame; **D.** Feature map of LiP-H8 destination clone - created with the Seqbuilder module of Lasergene (V7.1) to show the insert and resulting in a plasmid that is Cm sensitive and Am resistant; **E.** Colony PCR of recombinant pYES-DEST 52 plasmids with FC/RC primer pair (Lane 1 to 3 ~1.1kb amplicon); **F.** RE digestion of the recombinant pYES-DEST-52plasmid with KpnI (RE site in the LiP-H8 gene). Lane 1, Uncut recombinant pYES-DES-52 plasmid; Lane 2– KpnI digested pYES-DES52 recombinant plasmid (7.5kb).

advantages such as genetically stable expression host, eukaryotic specific post translational modifications and adaptation to low cost culture conditions to result in high biomass fermentation (Wang *et al.* 2004, Wang and Wen 2009). The amplified and sequenced full length LiP H8 gene was cloned into pENTR-D TOPO gateway cloning vector. The recombinant vector could be verified by RE digestion which yielded an inserted of around 1.1 kb and sequencing with the vector primers confirming the directional cloning (Figs 2B, C). The transfer of the full-length Lip H8 gene from the entry clone pENTR-D TOPO-Lip-H8 into the destination vector was performed using the clonase enzyme mix. The recombinant destination vector pYES-Lip was confirmed by colony PCR (Fig. 2E) and digestion with *KpnI* (RE site in the Lip-H8 gene) linearized the plasmid (Fig. 2F).

Correlations between the lignolytic ability of the microorganism to the decolourization of different dyes were reported (Glenn and Gold 1983, Pasti and Crawfod 1991). This ability of the fungi was employed in a rapid dye decolourization agar plate method to assess the oxidative

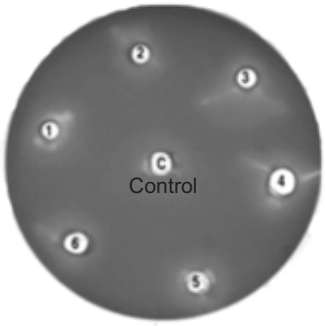

Fig. 3. Ingel- enzyme assay for expression of the recombinant Lignin peroxidase. Screening for extracellular enzyme production in the recombinant LiP-H8 destination colonies in agar plates supplemented with methylene blue dye. **C.** *E.coli* cells harboring pYES-DEST52 control vector; Wells 1-6 – LiP H8 destination clones. Note: Enzyme production indicated by the clearance of zone after the addition of H_2O_2.

biodegradation of lignin and utilized to screen the efficiency of new wood preservatives (Borokhov and Rothenburger 2000). Hence, in our study we used a similar method in which

the *E.coli* cells harbouring pYES LiP-H8 were added to wells punched in agar plates supplemented with methylene blue dye. The colonies were assessed for the oxidative property of the LiP leading to decolourize of the azo dye. The *E.coli* cells harbouring the pYES LiP- H8 could decolourize the methylene blue in the presence of H_2O_2 after 48 h, which was visible as a clear zone compared to the controls confirming an active expressible and functional clone (Fig. 3). This indicated the ability these recombinant clones to secrete functionally active lignin peroxidase. Further studies are required to conduct experiments for their bioremediation ability with different azo dyes and also applicability of this clone in degrading the complex plant polysaccharide matrix.

Nitrogen limiting culture conditions induced the secretion of lignin peroxidase by *Phanerochaete chrysosporium* as early as 2 days following culture which peaked on day 4. The full-length major isoform of lignin peroxidase (LiP-H8) could be amplified that was cloned to have an active expressible clone. This functional clone is capable of degrading methylene blue *in-vitro*. Further experiments on the bioremediation capability with different azo dyes, chemicals and plant polysaccharide matrix would open up an attractive and alternate utility of this functional clone.

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