Published in final edited form as:
Enzyme Microb Technol. 2008 August 5; 43(2): 205–213. doi:10.1016/j.enzmictec.2007.09.001.

REGULATION AND HETEROLOGOUS EXPRESSION OF P450 ENZYME SYSTEM COMPONENTS OF THE WHITE ROT FUNGUS *PHANEROCHAETE CHRYSOSPORIUM*

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Abstract

Phanerochaete chrysosporium is widely used as a model organism to understand the physiology, enzymology, and genetics of lignin degradation by white rot fungi and is known for its ability to metabolize and detoxify a wide range of environmental chemicals. Our pre-genomic efforts and the recent whole genome sequencing by the Joint Genome Institute of the US-DOE have revealed that this fungus carries a well developed P450 enzyme system, consisting of multiple P450 monooxygenases and a common P450 oxidoreductase. The entire P450ome of this organism comprises of ~150 cytochrome P450 monooxygenases, mostly arranged in gene clusters and classifiable into multigene families. Except for the structurally and functionally conserved fungal P450 families such as CYP51, CYP61, and CYP53, other P450 enzymes in this organism have largely unknown function and will require functional characterization. These new P450 enzymes may likely have roles in biodegradation activity and physiology of this ligninolytic fungus. Our pre- and post-genomic efforts to understand the functional role of P450 enzyme systems in *P. chrysosporium* have focused on the regulation of expression of the first identified family of P450 enzymes, the CYP63 family, and genome-wide regulation of the other P450 families using a custom-designed P450 microarray. The genomically-linked CYP63 member P450s were found to be differentially regulated under varying physiological and/or biodegradation conditions. Results on the heterologous expression of this family of monooxygenases in different prokaryotic and eukaryotic expression systems are presented and the inherent problems associated with the expression of these membrane proteins are discussed. Further, we report the expression and purification of the white rot fungal cytochrome P450 oxidoreductase (POR), the electron transfer component of its P450 enzyme system, required for P450 catalysis. The reported studies have uncovered the hitherto unknown regulatory aspects of the P450 enzyme system in *P. chrysosporium* and generated useful expression tools and knowledgebase to pursue further studies on functional analysis of the P450 contingent in this model white rot fungus.

Keywords

Cytochrome P450 monooxygenase; P450 oxidoreductase; *Phanerochaete chrysosporium*; White rot fungus; Biodegradation

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1. Introduction

The model white-rot fungus *Phanerochaete chrysosporium* is widely known for its inherent capacity to completely breakdown the plant cell wall polymer lignin as a part of the nature's carbon cycle and its ability to biodegrade or mineralize a wide range of toxic chemical pollutants such as petroleum hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), herbicides, pesticides, detergents, dyes, preservatives etc. [1,2]. Originally, the biodegradation ability in this organism was attributed to the presence of two classes of extracellular peroxidases, lignin peroxidases (LiPs) and manganese peroxidases (MnPs), in conjunction with multiple H_2O_2-generating enzymes, all of which are expressed under nutrient starvation (ligninolytic) conditions during secondary metabolism in this organism. However, it has been consistently shown by us and others that oxidation/degradation of several organic pollutants such as PAHs, BTEX compounds, alkyl benzene sulfonates etc. can occur even under peroxidase-suppressing (non-ligninolytic) conditions [3,4,5], indicating the role of other oxidative systems including P450 monooxygenases in this organism. In this context, the recently completed whole genome sequence [6] has now revealed that *P. chrysosporium* possesses an entire gamut of alternate or additional oxidation systems in its genome (http://genome.jgi-psf.org/whiterot), of which cytochrome P450 enzyme system is prominent, constituting about 1% of the coding genome. These pre-genomic and whole genome-based observations imply the involvement of multiple P450 monooxygenases in catalyzing the ligninolysis and the initial oxidation of various chemical compounds under low-nutrient (ligninolytic) and/or high-nutrient (non-ligninolytic) conditions. The current working hypothesis on the role of P450 enzyme system in lignin biodegradation in this white rot fungus is that these intracellular monooxygenases catalyze the subsequent oxidation of the peroxidase-depolymerized lignin derivatives leading to complete mineralization of lignin to CO_2.

Cytochrome P450 enzymes are heme-thiolate proteins that are known to catalyze the metabolism of a variety of exogenous and endogenous compounds in prokaryotes and eukaryotes. The typical eukaryotic P450 monoxygenase system contains a P450 monooxygenase and a P450 oxidoreductase (POR), both of which are normally membrane-associated. The whole genome sequence has revealed that the P450 monooxygenase system of *P. chrysosporium*, a lower eukaryotic organism, comprises of ~ 150 P450 monooxygenases and a P450 oxidoreductase. In this report, we present our pre-genomic and post-genomic efforts to analyze and characterize this elaborate intracellular monooxygenase system, both at the level of transcription and translation, with particular emphasis on the first identified CYP63 family of P450 monooxygenases in this fungus as well as purification of the P450 oxidoreductase. The information generated is expected to pave the way for future studies on catalytic analysis and role of the P450 monooxygenase system in ligninolysis and bioremediation in this white rot fungus.

2. Materials and Methods

2.1. Strains and culture conditions

P. chrysosporium strain BKM-F-1767 (ATCC 24725) used in this study was maintained on malt extract (ME) agar (Difco Laboratories, USA). *P. chrysosporium* cultures were grown as shaken cultures at 37 °C in defined low N medium (low N), high N medium (high N), or Malt extract medium (ME), as described elsewhere [4].

2.2. Transcriptional analysis by custom-designed P450 microarray and quantitative reverse transcription-PCR

For regulation studies using microarray or quantitative real time reverse transcription-PCR (RT-PCR) analysis, total RNA was extracted from the cultures harvested on day 4 [7,8]. Total

RNA for induction experiments using RT-PCR analysis was prepared from fungal cultures grown using a consecutive two day culturing protocol, with xenobiotic inducer added after 1 day of incubation, as described earlier [9,10,11]. Microarray slide printing (spotting), hybridizations, and scanning were performed at the Genomics and Microarray Laboratory of the University of Cincinnati following their protocols (http://www.microarray.uc.edu), as described elsewhere. Quantitative RT-PCR analysis was performed using gene-specific primers using the reaction conditions described elsewhere [10].

2.3. Heterologous expression in *E. coli*

The *P. chrysosporium* P450 system genes *pc-1*, *pc-3*, and *POR* were expressed in *E. coli*. The *pc-1* cDNA was custom-synthesized for codon optimization (BIO S&T, Inc., Canada). The cDNA of interest was cloned into the vector pET30a(+) (EMD Biosciences, Inc., USA) such that it was in frame with the N-terminal histidine tag. This construct was transformed into *E. coli* RosettaBlue(DE3) (EMD Biosciences, Inc., USA) cells. A transformed colony was then inoculated into 5 ml Luria Bertani (LB) broth containing antibiotics and grown overnight at 37 °C. This overnight grown culture was then transferred to 100 ml of LB broth and allowed to grow to an O.D660 of 0.5. Cultures were then induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) for an additional 4 h at 25 °C, 30 °C, and 37 °C. Protein extraction was done using CellLytic B bacterial cell lysis extraction reagent (Sigma, Inc., USA) per the manufacturer's protocol. The expressed protein was detected by Western blot analysis using anti-his antibody (PC-3 and POR) or protein-specific antibody (PC-1).

2.4. Heterologous expression in *Saccharomyces cerevisiae*

pc-1 cDNA was inserted into pYES2.1/V5-His-TOPO vector (Invitrogen Corp., USA) such that it was under the control of the *GAL1* promoter and was in frame with the C-terminal histidine tag. This construct was then transformed into Y300 yeast strain (*MATa ade2-1 trp1-1 ura3-1 leu2-3,112 his 3-11,15 can1-100*), kindly provided by Dr. Yolanda Sanchez of the University of Cincinnati. A single transformed colony was then inoculated into synthetic complete medium lacking uracil (SCD-ura) containing 2% glucose and incubated overnight. This culture was centrifuged and transferred into 600 ml SCD-ura medium containing 2% galactose so as to get an $O.D_{600}$ of 0.4. After incubation for 0, 4, 8, 12, 16, 20 and 24 h, the cultures were harvested by centrifugation and snap frozen. Microsomes were extracted by centrifugation at 1,00,000 × g for 90 min and the expressed P450 was detected by Western Blot analysis using the PC-1-specific antibody.

2.5. Heterologous expression in insect cell system

Sf9 (*Spodoptera frugiperda*) insect cells grown in serum-free media (SFM) to a cell density of 2×10^6 cells/ml were infected with recombinant Baculovirus containing *pc-1* cDNA at a multiplicity of infection of 3.0. The recombinant bacmid construct was generated as follows: the *pc-1* cDNA was cloned such that it was under the transcriptional control of the polyhedrin promoter, in pFASTBAC HTa (Invitrogen Corp., USA) cloning vector. This was followed by transformation into DH10Bac cells (Invitrogen Corp., USA). At 24 h post infection, hemin was added to the cells at a concentration of 3 μg/ml and the cells were harvested at 24, 48, 72 and 96 hr post addition of hemin followed by isolation of cell extracts. Microsomes were extracted from these cell extracts by centrifugation at 1,00,000 × g for 90 min. A total of 100 μg of microsomal protein for each time point was run on 10% SDS-PAGE followed by blotting onto nitrocellulose membrane, and detection of the expressed protein using Western blot analysis as described above.

2.6. Purification of the expressed POR

Purification of the white rot P450 oxidoreductase protein expressed in *E. coli* was done by passing the Triton X-100 detergent-solubilized total protein extract through Ni-NTA agarose column. Washing and elutions were done using increasing concentration of imidazole (5 mM – 80 mM). The expressed and purified POR was detected using anti-POR antibody. Functional activity was determined by performing a cytochrome C reductase assay [12].

3. Results and Discussion

3.1. P450ome of *P. chrysosporium*

The whole genome sequence of *P. chrysosporium* and the initial annotation revealed an initial estimated number of 148 P450 monooxygenase genes [6]. This turned out to be the highest number known till that date among the fungal genomes. The P450 Nomenclature group (http://drnelson.utmem.edu/Genome.list.htm.) predicted a total of 163 P450 sequences of which 126 were full-length or near full length. Using these predictions, we reported the occurrences of gene clustering of the P450 genes into 26 clusters based on overall homology [7]. Our phylogenetic analysis showed that the 126 full-length/near full-length genes could be grouped under 12 families and 23 subfamilies [13,14]. The grouping was based on the amino acid sequence similarity using the existing criteria of less than 40% similarity defining a family and less than 55% similarity defining a subfamily. The 12 families under which these P450 genes could be classified are: CYP64, CYP67, CYP503, CYP58/53, CYP63, CYP505, CYP614/534, CYP617/547, CYP5031/CYP547, CYP51, CYP61, and CYP62. Among these, the CYP64 family consists of the highest number of P450 genes (54 genes). Due to the ever expanding superfamily of P450 genes, it has been getting increasingly difficult to classify P450 proteins at the family level based on evolutionary and functional relationships. This has led to the introduction of the term “clan”, which is based on relationships that are beyond family level classifications. Typically, a clan represents a cluster of P450 families across species. Clan-level comparison revealed that the 12 *P. chrysosporium* P450 families had resemblances to 11 fungal P450 clans. This suggested that progenitors of the P450 genes in *P. chrysosporium* were probably acquired from a common ancestor [13]. In addition to the family- and clan-based classification, genome-wide structural analysis also revealed that the P450 genes are present as clusters on the genome. As high as 16 P450 gene clusters were identified with the highest number belonging to the CYP64 family [13].

3.2. CYP63 gene family

CYP63 gene family, the first identified P450 family in white rot fungus, has been the focus of research since the cloning of the first two members of this family in our laboratory [9]. This multigene P450 family consists of seven members designated as *pc*-1 through *pc-7*. These seven genes are structurally related to each other to different extents at the amino acid levels based on their percentage of similarity or divergence as shown in Table 1. The coding sequence in these genes varies from a minimum of 1713 bp in *pc-6* to a maximum of 1809 bp in *pc-3* [13,14], and is interrupted by multiple small introns in a conserved manner. The typical sequence motifs, namely I-helix, K-helix, and HR2 region were also quite conserved. In our pre-genomic cloning efforts, the first three genes *pc-1* (CYP63A1), *pc-2* (CYP63A2), and *pc-3* (CYP63A3) of this family were shown to be tandemly linked [9] and were subsequently localized on the same scaffold in the genome [6]. *pc-1* was found to be alternatively spliced, based on the two splice variants detected in cultures grown under nutrient-rich conditions. Sequencing of the 5’ proximal part of the cDNA sequence revealed five introns in variant 1 as compared to four introns in variant 2 [9]. Two other genes of this family, *pc-5* (CYP63B1) and *pc-6* (CYP63B2) were subsequently found to be tandemly linked but on a different scaffold in the genome. The other two genes in this family, *pc-4* (CYP63A4) and *pc-7* (CYP63C1) are localized on different scaffolds in the genome.

3.3. Native (transcriptional) expression of P450 enzymes of *P. chrysosporium* under varied physiological and biodegradation conditions

3.3.1. Physiological regulation of expression of P450 monooxygenases

3.3.1.1. Global P450 gene regulation: We developed the first custom-designed 70 mer oligonucleotide chip [7,8] based on the predicted P450 genes. Our microarray analysis using this chip revealed that all the P450 genes are constitutively expressed, both under ligninolytic (low N) and non-ligninolytic (high N) conditions. However, 27 of the P450 genes were found to be differentially regulated between the two nutrient conditions. Of the 27 genes, 23 genes were upregulated under high N conditions, where as 4 genes showed upregulation under low N conditions [7,8]. Our phylogenetic analysis of the whole P450ome had revealed that P450 genes could be clustered into 16 genomic clusters. Of these, 10 clusters showed non-assortative regulation of expression of its member genes suggesting divergence of function associated with the encoding P450 enzymes [8]. Specifically, the microarray analysis also revealed that *pc-1* expression was downregulated to an extent of 0.48-fold in high N medium as compared to low N medium. On the other hand, expression of *pc-2* was 2.26-fold upregulated in high N condition as compared to low N condition [8, Table 2]. Expression of *pc-3* remained at a steady state under both the nutrient conditions.

3.3.1.2. CYP63 specific gene regulation: Our initial transcriptional analysis (pre-genomic) using quantitative real time RT-PCR had revealed that *pc-1* expression was highest in the defined low nitrogen (low N) medium as compared to high N (high nitrogen) or malt extract (ME) medium [9]. In contrast, *pc-2* was found to be expressed at an overall higher level under high N conditions. These differential expression patterns in response to nutrient levels were found consistent with our subsequent global analysis using the first custom-designed P450 microarray described above [8]. Time course transcriptional analysis of the CYP63 genes by quantitative real time RT-PCR revealed peak expression on day 4 for both *pc-1* and *pc-2* under defined low N and high N conditions, respectively [10]. Like *pc-1*, *pc-3* expression was found to be higher in the defined low N medium as compared to the high N and ME medium [11].

Effect of temperature was studied for the three tandemly linked genes (*pc-1*, *pc-2*, and *pc-3*) in low N medium. *pc-1* expression was found to be higher at 37 °C than at 22 °C, where as *pc-2* and *pc-3* showed no significant difference between these temperatures [10,11,Table 2]. Oxygenation seemed to have an overall positive impact on expression of all three genes [10, 11,Table 2]. Low N cultures using different carbon sources showed varied levels of expression of *pc-1*. Highest level of expression was found in glucose, followed by sucrose or raffinose, starch, and carboxy methyl cellulose (CMC). Growth on sucrose and raffinose as sole carbon sources showed similar levels of *pc-1* expression [10,Table 2]. Expression of *pc-1* under high N conditions was found to be dramatically high as observed in 4-day-old cultures grown on glucose as sole carbon source. *pc-2* expression was found to be relatively higher when grown on sucrose or raffinose as sole carbon source under low N conditions as compared to glucose, starch, or CMC [10]. *pc-3* expression was found to be higher when grown with starch as the sole source of carbon in low N cultures [11, Table 2]. These observations led to the conclusion that although belonging to the same family of cytochrome P450 proteins, and despite being structurally conserved and tandemly arranged on the same scaffold, the three genes *pc-1*, *pc-2*, and *pc-3* are independently regulated by nutrients (nitrogen and carbon) and other physiological conditions (temperature).

3.3.2. Xenobiotic induction of P450 monooxygenases

3.3.2.1. Induction by industrial/environmental chemicals: It is well known that several inducers for eukaryotic P450 monooxygenases can also be the substrates that these enzymes can oxidize [15]. This prompted us to study the induction pattern of the three CYP63 member genes (*pc-1*, *pc-2*, *pc-3*) in response to several xenobiotic inducers, with an aim to identify their

corresponding substrates. *P. chrysosporium* cultures grown in ME medium (high N and high C) were induced with 42 different xenobiotic compounds that represented a wide range of chemical structures, including aliphatics, aromatics, polyaromatics, alkyl-substituted aromatics, alicyclics, and lignin derivatives. Classical eukaryotic P450 inducers (phenobarbital, estradiol) were also used for reference purposes. We selected the ME medium for studying the induction pattern firstly, because peroxidase expression (LiP and MnP) is known to be suppressed under these conditions, and secondly, P450-mediated oxidation of xenobiotics has been reported under these culture conditions earlier [3,4,5]. Majority of the compounds tested in this study were included because of their known degradability by *P. chrysosporium* in laboratory studies [2]. While *pc-1* was shown to be highly induced by monocyclic aromatics (alkyl-substituted aromatics, hydroxylated aromatics) and lower molecular weight PAHs (2–4 rings), *pc-2* was induced by high molecular weight PAHs (4–5 rings), and environmentally recalcitrant chemicals DDT, and long-chain alkylphenols [10,Table 2]. Our observation that both *pc-1* and *pc-2* are induced by alkyl-substituted aromatics could be attributed to the fact that a conserved motif (RDTTAG) in the I-helix of these genes was similar to that present in other alkane-hydroxylating P450 proteins [9]. This further supported our hypothesis that inducers for at least some of the P450 enzymes could likely be their substrates as well. PC-3, which also falls under the same family of P450 monooxygenases was also expected to show similar induction pattern in response to xenobiotic substances, since it shared a close overall aa homology of 85.2% with PC-2 and 58.9% with PC-1 (Table 1), and also shared the conserved putative substrate-binding domain in the helix-I. In fact, *pc-3* was also found to be induced in response to linear alkanes similar to *pc-2*, and by simple aromatics similar to *pc-1* in our subsequent analysis. Polycyclic aromatic compounds were however shown to induce all the three P450 genes, albeit to varying extents [10,11, Table 2].

3.3.2.2. Induction by lignin derivatives: In addition to the individual xenobiotic chemicals, we also tested the induction of CYP63 genes with the following commercially available lignin derivatives: lignin alkali, lignin alkali carboxylated, lignin alkali 2-hydroxy propyl ether, and lignosulfonic acid. Interestingly, both *pc-1* and *pc-2* were induced several folds with lignosulfonic acid [10,Table 2]. *pc-3*, however, responded only to lignin alkali [11, Table 2]. These compounds are used as dispersing agents in industrial applications. These observations added further credibility to the current working hypothesis that lignin after depolymerization by the non-specific peroxidase enzyme system can be internalized by the fungal hyphae and the lignin derivatives can then induce the P450 enzymes for subsequent breakdown of the lignin during its mineralization to CO_2.

3.4. Heterologous expression of the cytochrome P450 monooxygenases of *P. chrysosporium*

The observed differential regulation pattern of the CYP63 family of proteins in response to physiological and biodegradation conditions, and their responses to specific chemical compounds provided an impetus for us to study their role in degradation of the identified likely xenobiotic substrates. An innate problem associated with studying the role of cytochrome P450 enzymes in the native host (*P. chrysosporium*) is their redundancy coupled with the difficulty in purifying the P450 monooxygenase of interest from the pool of ~150 P450 proteins that this fungus expresses. Hence, in order to pursue this goal, we undertook heterologous expression studies on selected P450 system genes in our laboratory. As has been the case with peroxidase genes, heterologous expression of P450 monooxygenases revealed inherent problems, presumably because of the high GC composition and divergent codon usage in this organism. This necessitated codon optimization and/or use of different expression systems to achieve translation. A brief account of the different attempts made and their outcomes in the different expression systems is discussed in the following sections.

3.4.1. Expression in *E. coli*—Our initial efforts to heterologously express PC-1 (CYP63A1) enzyme in *E. coli* strain BL21(DE3) did not yield the translation product. The plasmid vector system used in this effort was the *E. coli*-yeast shuttle vector lambda YES. Our subsequent efforts to express other proteins from this CYP family namely PC-2 and PC-3 in this vector system also did not yield the desired product. Differences in codon usage could be one possible answer to the general inability of *E. coli* to express proteins from eukaryotic systems like *P. chrysosporium* efficiently. It is well known that differences in codon usage can result in reduced translation due to the lack of specific tRNAs required by the translation machinery in such systems [16,17]. We therefore codon optimized one of the CYP63 genes (*pc-1*) and got its cDNA custom-synthesized (Bio S&T Inc., Canada) in a way that it would be recognized both in the yeast *Saccharomyces cerevisiae* and in *E. coli*. We followed the common rules for codon optimization; all alanines were replaced by GCT, arginines by AGA, asparagines by AAT, aspartic acid by GAT, cysteine by TGT, glutamine by CAA, glutamic acid by GAA, glycine by GGT and GGA, histidine by CAT, isoleucine by ATT and ATC, leucine by TTA, CTA, and TTG, lysine by AAG, phenylalanine by TTC and TTT, proline by CCA and CCT, serine by TCT, threonine by ACT, tyrosine by TAT, and valine by GTC and GTT. Sequence variation introduced in the "synthesized *pc-1* gene" as compared to the "native *pc-1* gene" is shown in Fig. 1. Among the incubation temperatures tested, 37 °C supported the highest levels of expression (Fig. 2A). We therefore used these conditions for our further expression studies. While the expression levels were high, our analysis revealed that most of the protein remained as inclusion bodies, which are aggregates of misfolded protein (Fig. 2B). Formation of such inclusion bodies is a common phenomenon that has been observed during expression of several eukaryotic proteins, especially membrane proteins in *E. coli* [18,19].

In contrast, PC-3 was successfully expressed in *E. coli* in soluble form using a similar expression system [11]. However, the amount of protein expressed was not as abundant as that of the PC-1. This could be explained by the codon optimization effect in *pc-1* as compared to *pc-3* that had the original gene sequence. Expression of these two genes demonstrated that P450 expression in BL21-derived strains like RosetteBlue(DE3) was superior to that in the original strain. Although, protein expression was observed for both PC-1 and PC-3, the expressed proteins did not yield a typical P450 spectrum. Low expression levels of these proteins could be the likely reason for undetectable spectrum. Our initial efforts to refold the otherwise abundant misfolded PC-1 protein also did not yield the desired results.

3.4.2 Expression in yeast—Considering the constraints in *E. coli* system, we decided to attempt expression of the synthesized *pc-1* gene in a eukaryote using *Saccharomyces cerevisiae* expression system (see Methods section). Western blot analysis revealed that microsomal extracts prepared from the cells of a transformant showed PC-1 expression after 12 h of induction with galactose (Fig. 3a). However, the microsomal fraction did not yield the detectable P450 spectrum possibly because of the low level of expression.

3.4.3 Expression in Baculoviral expression system—Baculoviral expression systems are increasingly being used for heterologous expression of cytochrome P450 enzymes from plants and animals. This expression host has proven to be a useful system especially for proteins which need post-translational modifications e.g. glycosylation. Several P450 enzymes from higher eukaryotes (humans, mouse, bovine, and plants) have been shown to be functionally produced in this heterologous expression system. We therefore attempted to express PC-1 in a commercially obtained insect cell expression system (Invitrogen Corp., USA), as described under Methods section. Western blot analysis using anti-PC1 polyclonal antibody raised against a PC-1-specific peptide detected PC-1 expression in this system starting from 24 h post addition of hemin (Fig. 3B). However, the problem associated with observation of a typical soret peak at 450 nm still remained indicating that the expressed protein concentration may not be adequate for such analysis.

Based on previous studies, it is apparent that *P. chrysosporium* proteins are not so easy to express in common heterologous hosts. Especially, attempts to express lignin peroxidases in hosts such as *E. coli*, yeasts, and insect cells have met with similar difficulties and variable success [20,21,22,23,24]. Nevertheless, our attempts to express white rot P450s have led to the following conclusions. First, there is an extreme codon bias as shown by increased expression of PC-1 in the bacterial system after codon optimization. This problem could also be solved in part by using improved recipient derivative strains that possess additional plasmids encoding the rare tRNAs required for expression of eukaryotic proteins. Second, although these fungal P450 proteins show expression to a certain extent in eukaryotic systems like *Saccharomyces cerevisiae* (although by not all strains) and baculoviral systems, they do not show typical P450 spectrum possibly because they do not undergo the correct folding pattern either due to the native environment of these heterologous systems or due to the lack of incorporation of heme into the protein. Alternately, the level of expression of these proteins in the eukaryotic systems was probably not high enough to allow detection of the typical P450 soret peak. This problem has been commonly observed in the past with baculoviral system [24]. These issues point to the need for more intensive efforts towards protein refolding or towards strategies for increasing the levels of expression in these heterologous systems.

3.5. Heterologous expression of white rot fungal P450 oxidoreductase in *E. coli*

In contrast to the cytochrome P450 monooxygenases, the P450 oxidoreductase gene (*POR*) was successfully expressed in the bacterial expression system (*E. coli*). Oxidoreductases are electron transferring proteins that transfer electrons from NADPH to P450 monooxygenases, which in turn catalyze the oxidation of chemical compounds. The POR protein expressed in *E. coli* was detected both by Western blot analysis using anti-POR antibody as well as by performing cytochrome C reductase assay [12]. Purification of the expressed POR protein was done by passing the extract through Ni-NTA agarose column (Fig. 4). We were able to purify the POR protein up to 18-fold in comparison to the crude extract. The purification led to an increase in the specific activity of the protein from 40.7 U/mg to 732.95 U/mg (Table 3).

4. Conclusions

Our studies reported herein on the P450 enzyme system in *P. chrysosporium* have led to an understanding of the regulation of expression of the P450 enzymes in general and CYP63 enzymes in particular in this model white rot fungus. Global expression analysis showing constitutive expression of all P450 enzymes in a broad nutrient range (low to high) implies their wider involvement in the catalytic activity of this organism. Differential upregulation of certain P450 enzymes under nutrient-limited and nutrient sufficient conditions points to their likely specific role under ligninolytic and non-ligninolytic conditions, respectively. The transcriptional expression patterns of the individual members of CYP63 family showed that these P450 genes are independently regulated despite being structurally conserved and tandemly linked in the genome. A substrate-specificity among the CYP63 enzymes is indicated based on their differential pattern of induction by different xenobiotic chemicals/substrates and lignin derivatives. A more comprehensive understanding of the transcriptional expression of these important biocatalysts in response to individual classes of xenobiotics and lignin-derived compounds could help in designing better conditions for bioremediation of diverse toxic chemicals found in the environment and ligninolysis/bioconversion processes. The heterologous enzyme expression studies on components of the P450 enzyme system of *P. chrysosporium* have yielded a functionally active purified P450 oxidoreductase for use in future catalytic analysis. Efforts on CYP63 P450 monooxygenases, that yielded low levels of expressed proteins or inactive proteins, have brought out the key problems encountered in expressing these membrane proteins in non-native systems. Future efforts will focus on using other species and strains of yeast and filamentous fungal hosts as well as toward refolding of

the bacterially expressed proteins to obtain sufficient active white rot fungal P450 proteins for catalytic analysis and applications. Considering the unlimited oxidizing potential of *P. chrysosporium*, it is conceivable that its large P450 contingent may represent an unusually high number of potentially important industrial enzyme biocatalysts. This catalytic versatility may justify the time and effort expected to delineate the function of these enzymes from this organism.

Acknowledgements

This work was supported by the NIH's National Institute of Environmental Health Sciences (NIEHS) grant R01-ES10210 (J.S.Y.). We thank Harshavardhan Doddapaneni for insightful discussions during the course of this study.

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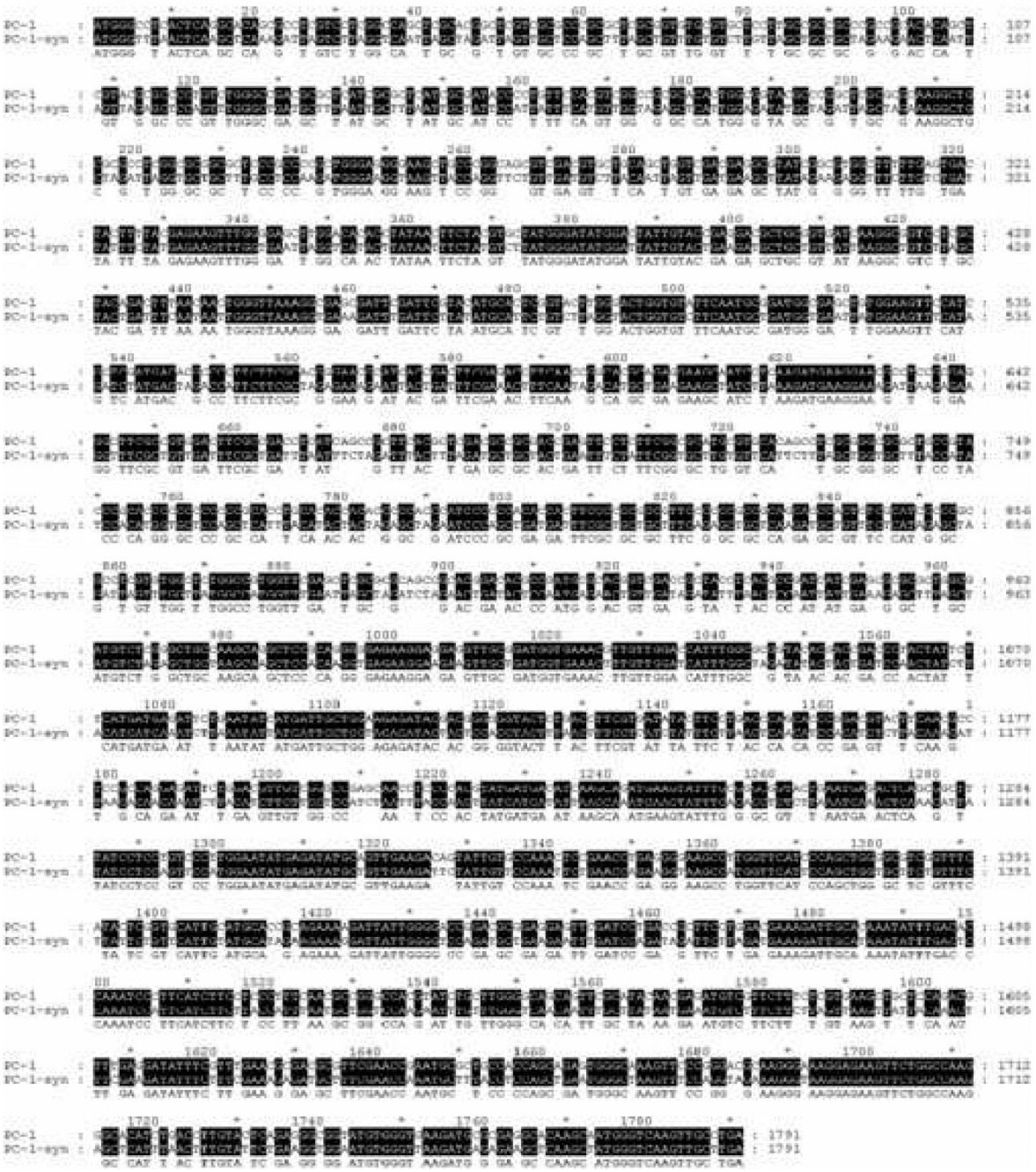

Fig. 1.

Sequence alignment of the native sequence (*pc*-1) and the codon-optimized sequence (*pc*-1-syn) of CYP63A1 cDNA of *P. chrysosporium*. MegAlign 5.05 and GeneDoc Version 2.6.002 softwares were used for the alignment. Altered bases are not highlighted.

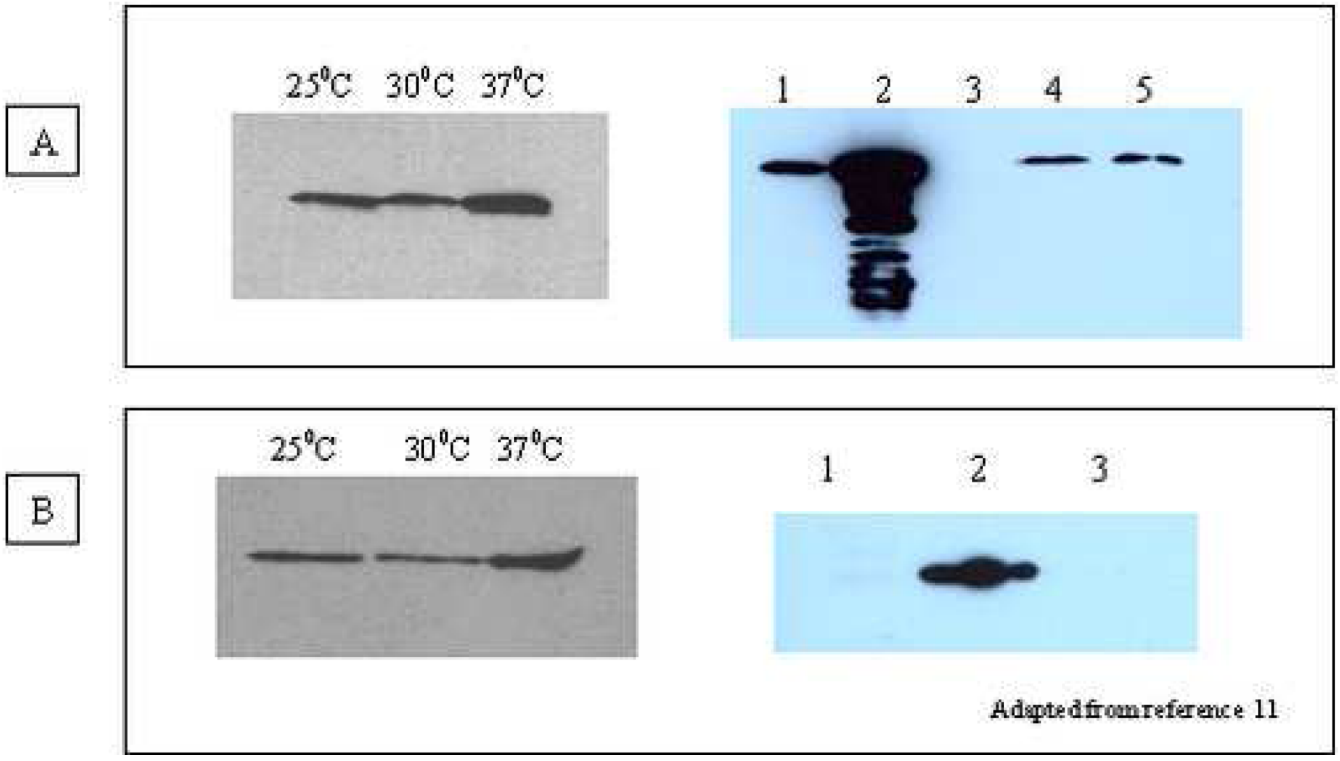

Fig. 2.

Heterologous expression of the white rot fungal P450 monooxygenases PC-1 and PC-3 in *E. coli*.

A: Left panel, Effect of temperature on PC-1 expression. Right panel, Expression of PC-1 in *E. coli* at 37 °C. 1 – uninduced inclusion bodies, 2 – induced inclusion bodies, 3 – vector control, 4 – induced soluble fraction, 5 - uninduced soluble fraction.

B: Left panel, Effect of temperature on PC-3 expression. Right panel, Expression of PC-3 in *E. coli* at 37 °C. 1 – uninduced soluble fraction, 2 – induced soluble fraction, 3 – vector control.

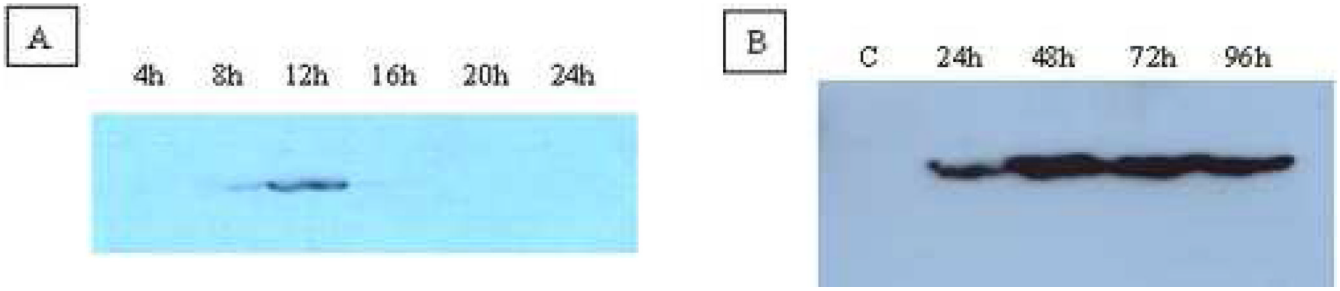

Fig. 3.

Heterologous expression of the white rot fungal P450 monooxygenase PC-1 in eukaryotic expression systems.

A. PC-1 expression in *Saccharomyces cerevisiae*. Yeast cultures grown in SCD-ura and induced with 2% galactose for varying incubation times (0, 4, 8, 12, 16, 20, and 24 h) were harvested and the expressed protein was detected using anti-his antibody.

B. PC-1 expression in Baculoviral cell line. Sf9 cells expressing PC-1 protein were harvested after 24, 48, 72, and 96 h post addition of hemin and the expressed protein was detected using anti-PC-1 antibody.

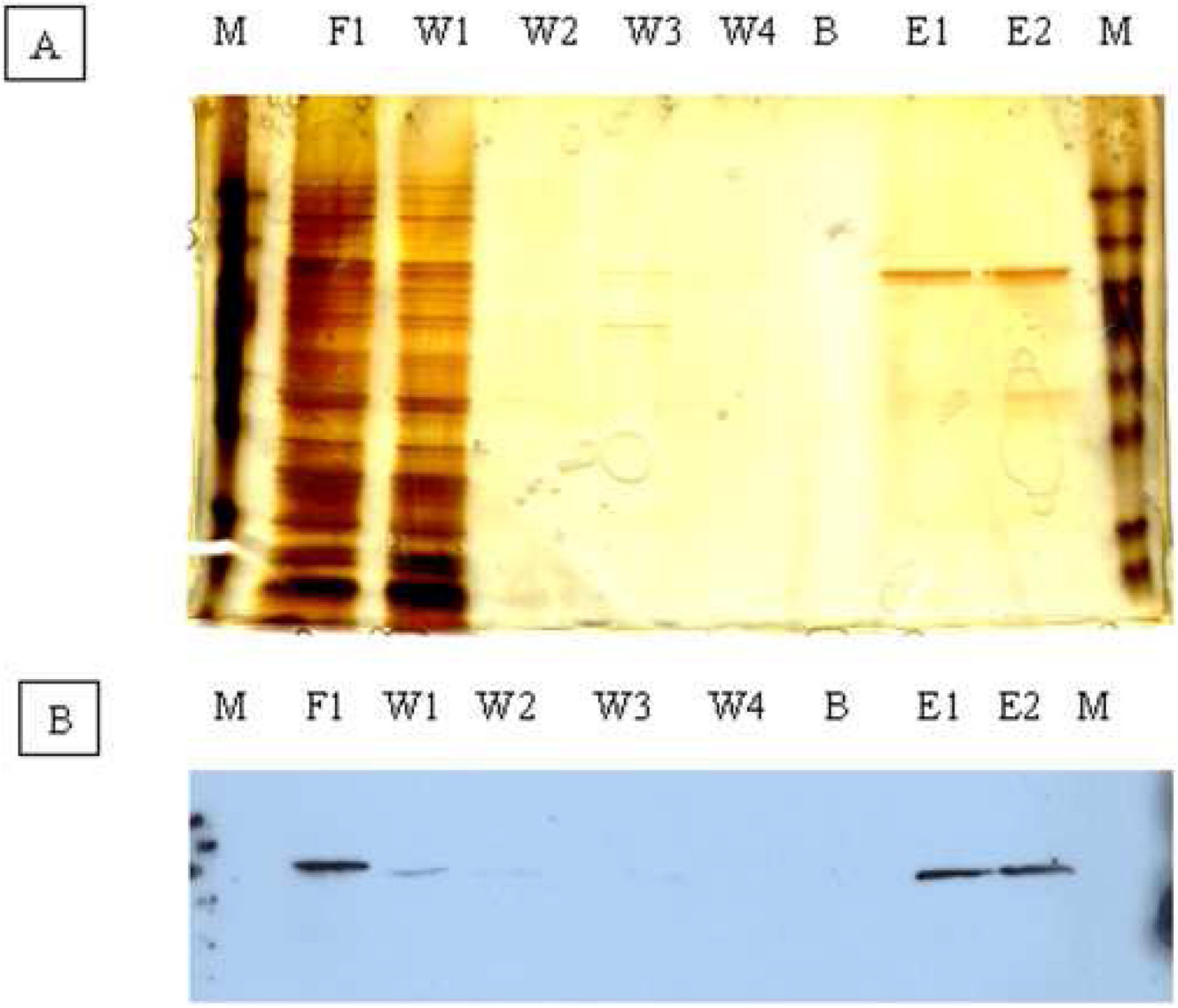

Fig. 4.

Heterologous expression of the white rot fungal P450 oxidoreductase (POR) in *E. coli* and its purification.

A. Total protein extract was passed through Ni-NTA column followed by washing and elution with increasing concentrations of imidazole. An equal volume was loaded on a 10% SDS-PAGE gel followed by silver staining of the gel.

B. Western blot analysis using anti-POR antibody.

M, Marker; F, Flowthrough; W, Washing; E, Elutions.

Table 1

Deduced protein sequence distances among the members of CYP63 family as determined using MegAlign 5.05

Percent Similarity →

Divergence ↓

	PC-1	PC-2	PC-3	PC-4	PC-5	PC-6	PC-7	
PC-1	***	58.9	58.9	49.5	35.2	34.7	32	**PC-1**
PC-2	57.5	***	85.2	47.6	34.4	33.5	33.5	**PC-2**
PC-3	57.5	16	***	48.6	34.9	33.3	34	**PC-3**
PC-4	80.5	82.2	79.2	***	37.4	36.4	33.3	**PC-4**
PC-5	128.3	128	129.9	125.1	***	76.9	42.3	**PC-5**
PC-6	125.6	127.7	129.9	118.5	27	***	42.4	**PC-6**
PC-7	143	135.9	133.9	140.9	93	97.4	***	**PC-7**
	PC-1	**PC-2**	**PC-3**	**PC-4**	**PC-5**	**PC-6**	**PC-7**	

Values shown in horizontal axis represent percent similarity. Values shown in vertical axis represent divergence in terms of phylogenetic distance.

Table 2

Effect of different physiological conditions and xenobiotic treatments on induction of the tandemly-linked P450 members of the CYP63 family

Variable	Fold expression		
	pc-1	***pc-2***	***pc-3***
Growth condition			
High N vs Low N	0.48 ± 0.18 *	2.26 ± 0.23 *	1.12 ± 0.07 *
37 °C vs 22 °C (Low N)	2.13 ± 0.04	0.84 ± 0.04	0.95 ± 0.06
O_2 vs Air (Low N)	3.67 ± 0.10	17.69 ± 2.80	2.17 ± 0.48
Carbon source			
Sucrose vs Glucose	0.77 ± 0.03	2.01 ± 0.02	0.80 ± 0.00
Raffinose vs Glucose	0.71 ± 0.01	2.43 ± 0.15	0.77 ± 0.19
Starch vs Glucose	0.37 ± 0.04	1.35 ± 0.19	2.00 ± 0.05
CMC vs Glucose	0.27 ± 0.07	1.63 ± 0.00	0.78 ± 0.02
Xenobiotics			
Aliphatics	0.78 ± 0.20 – 5.11 ± 0.16	0.31 ± 0.03 – 31.48 ±0.32	0.07 ± 0.00 – 4.85 ± 0.15
Aromatics	1.36 ± 0.19 – 6.24 ± 0.24	0.64 ± 0.13 – 1.62 ± 0.06	1.50 ± 0.13 – 2.73 ± 0.00
Poly aromatics	1.38 ± 0.07 – 6.27 ± 0.48	1.02 ± 0.12 – 6.02 ± 1.39	0.93 ± 0.44 – 2.87 ± 0.09
Alkyl-substituted aromatics	0.34 ± 0.21 – 6.18 ± 1.14	0.76 ± 0.08 – 23.63 ± 7.5	0.01 ± 0.00 – 2.21 ± 0.11
Alicyclics	0.27 ± 0.32	0.24 ± 0.06	0.39 ± 0.02
P450 inducers	0.64 ± 0.41 – 4.97 ± 0.65	3.69 ± 0.31 – 3.81 ± 0.68	1.00 ± 0.22 – 1.19 ± 0.04
Lignin derivatives	0.89 ± 0.46 – 8.61 ± 1.35	0.72 ± 0.45 – 4.56 ± 2.31	1.69 ± 0.02 – 2.77 ± 0.09

Values given are means ± standard deviations obtained from quantitative real time RT-PCR data. Data compiled from references 4,7,8,9,10.

* indicates values obtained from custom P450 microarray experiment.

Table 3

Purification of the recombinant white rot fungal POR heterologously expressed in *E. coli*, monitored in terms of specific activity, yield, and fold purification

	Total Volume (ml)	Protein (mg/ml)	Total Protein (mg)	Activity (U/ml)	Total Activity (U)	Specific Activity (U/mg)	Yield (%)	Purification (X)
Crude extract	50	1.2	60	48.85	2442.5	40.7	100	1
Purified protein	3	0.22	0.66	161.25	483.75	732.95	19.8	18