Expression Profiles of Fungal Cytochrome P450 Genes: A Mini Review

K.H.M. Nazmul Hussain Nazir[1]* and Mian Muhammad Awais[2]

[1]School of Basic Studies, Yeungnam University, Gyeongsan 712-749, Republic of Korea.
[2]Department of Pathobiology, Sub Campus Jhang,
University of Veterinary and Animal Sciences, Lahore, Pakistan.

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The authors describe the updates on expression of cytochrome P450 genes of fungi (mold and yeast) at mRNA level aiming to facilitate biotechnological application of these versatile biocatalysts in the industry for a series of applications like drug discovery, xenobiotic detoxification and/or bioremediation. This mini-review highlights how to solve the problems that may arise during studying expression profiles of P450s in liquid media, being the crucial step for stepping forward with the genes. Present knowledge on steps to be cared while conducting experiments for P450 clone library construction are summarized. Also, experimental evidences on expression profile of P450 genes in yeast using liquid media have been focused.

Key words: Cytochrome P450, Transcriptome, Liquid culture medium, mRNA, yeast, mold.

Cytochrome P450 enzymes (CYPs), a superfamily of heme-containing monooxygenases, are found throughout all the biological kingdoms including prokaryotes (archaea, bacteria), lower eukaryotes (fungi, insects), and higher eukaryotes (plants, animals and humans) (Nelson, 2009). CYPs play an important role in the metabolism of a wide variety of endogenous and xenobiotic compounds by catalyzing regio- and stereo-specific monooxygenation with an oxygen atom generated from molecular oxygen. Having a vital role in drug and xenobiotic metabolism and detoxification, mammalian CYPs have been studied extensively (Yamazaki, 2000; Inouye *et al*., 2002; Vrba *et al*., 2004; Shimada, 2006; Zhang *et al*., 2006; Warner *et al*., 2009; Abu-Bakar *et al*., 2012; Rozhon *et al*. 2013). On the other hand, CYPs from bacteria, yeasts and fungi have also been well studied for the biosynthesis of economically valuable consumer products, and in the detoxification and biodegradation of a broad spectrum of environmental chemical pollutants (Kelly *et al*., 1997; van den Brink *et al*., 1998; Lamb *et al*., 2000; Kelly *et al*., 2003; Seth-Smith *et al*., 2008; Nazir *et al*., 2011).

In recent years, a large-scale divergence of P450s has been explored through a series of genome projects (Nelson *et al*., 2011; Deng *et al*., 2007; Kelly *et al*., 2009; Park *et al*., 2008). The wide diversification of fungal P450s conferred survival in various environmental conditions by meeting various metabolic requirements. More than 12,000 P450 genes are named across all kingdoms of life (Nelson and Nebert, 2011). Currently, using computational annotation, 2487 species of fungal P450s have been identified and assigned to 399 families (Nelson *et al*., 1993). Besides bioinformatics studies, experimental approaches are also necessary for practical applications of these CYPs. In recent years, catalytic functions of many of these fungal CYPs have been explored (Subramanian *et al*., 2008; Sayed *et al*., 2010;

* To whom all correspondence should be addressed.
E-mail: nazir@ynu.ac.kr, nazirbau@gmail.com

Table 1. Oligonucleotides used for the PCR amplification of CYP52 genes from *C. albicans* and *C. tropicalis*

Yeast and CYP	Forward (5'-3')	Reverse (5'-3')
C. albicans		
CYP52A21	CCCACTAGTATGTTAGAAGAA ATCTCTTTTGAG	CCCGAGCTCCTAATCAACTTTAATA GGAACACC
CYP52A22	GGGACTAGTATGATGTCCCCCTC GCTTGTTCAC	GGGGAGCTCTTAATACATTTCAACA TTTGCCCC
CYP52A23	CCCACTAGTATGAGTGTTTCAGACA TTGTCCAA	GGGGAGCTCTTAATACATTTCAACAT TAGTACC
CYP52A24	GGGACTAGTATGTCCTCAACTACATT CAACGAT	GGGGAGCTCTCACATTTTCTTCAAGTT TTCAAA
CYP52C3	GGGACTAGTATGTTAATTTTATTACC TATTGCT	GGGGAGCTCCTAATCAACTTTAATAG GAACACC
C. tropicalis		
CYP52A1	CCCACTAGTATGTCGTCGTCTCCAT CTATTG	GGGGAGCTCTTAATACATTTGTA CTTCAG
CYP52A12	CCCACTAGTATGGCCACACAAGAAA TTATTG	GGGGAGCTCTTACATTTTAACGA AAACAC
CYP52A14	CCCACTAGTATGAGTATTCAAGATA TTGTTG	GGGTTAATTAACTAATACATTT GAATGTTGG
CYP52A17	CCCACTAGTATGATCGAACAAGTTGT TGAATAC	GGGTTAATTAACTAATCGATCTTG ACAATAGTTC
CYP52A20	CCCACTAGTATGATTGAACAGGTTTTA CATTATTG	GGGGAGCTCTTAATCCATCTT GACAAGCG
CYP52B1	CCCACTAGTATGTCATTAACAGAAAC AACTG	GGGGAGCTCTTATAATCTATG GAACTTGA
CYP52C1	CCCACTAGTATGTATCAATTATTTTGT TTTCTTG	GGGGAGCTCCTAGATAAAA CAAACGTTA

Hirosue *et al*., 2011; Nazir *et al*., 2011; Ide *et al*., 2012). In addition to the crucial biological roles of CYPs, utilization of their catalytic functions is of great interest in the biotechnology sector (Guengerich *et al*., 2002; Chang *et al*., 2007; Gillam *et al*., 2008; Urlacher *et al*., 2006). However, as the starting step aiming forward to wide application of these CYPs, expression of these P450 genes in a suitable media is crucial. Several media have been used for expressing full-length P450s. In this mini-review, we focused on the critical steps needed to be cared during experiments with expression profiles of P450s in mold. Also, for the first time, we validated the expression profile of CYPs in yeasts to find out a suitable media.

Different types of media for P450 expression

The genes, involved in secondary metabolic systems in fungi, are very likely to be affected by cultivation conditions (Tamano *et al*., 2008; Machida *et al*., 2005). A number of media like YPD (Nazir *et al*., 2010), complete medium (CM) (Pedrini *et al*., 2010), minimal medium (MM) (Pedrini *et al*., 2010), Czapek-Dox broth with casamino acids (MCB), Czapek-Dox broth with added yeast extract (CYB), yeast extract sucrose (YES) (O'Callaghan *et al*., 2003, O'Callaghan *et al*., 2006) and synthetic nitrogen-rich or nitrogen-limited media (Nazir *et al*., 2010; Ide *et al*. 2012) were used for studying P450 expression profiles. In *Aspergillus oryzae*, when it was grown in YPD medium or synthetic liquid culture medium under nitrogen-rich conditions, many P450 genes were not expressed except some housekeeping genes such as CYP51 and CYP58. On the other hand, a series of P450 genes in *A. oryzae* were strongly expressed when it was grown in a synthetic liquid culture medium under nitrogen-limited conditions. The genes of the family CYP51, known as sterol 14α-demethylase cytochrome P450s, are highly conserved across diverse organisms (Lepesheva and Waterman, 2007). The CYP58 family is also considered as important for fungal cells as a wide variety of fungal species possess homologous genes (Hohn *et al*., 1995). However, transcriptional regulation of fungal

P450s responds to nitrogen limitation or starvation, which has been shown in different filamentous fungus like *A. oryzae* (Nazir *et al*., 2010), *Coriolus versicolor* (Ichinose *et al*., 1999), *Phanerochaete chrysosporium* (Matsuzaki and Wariishi, 2004) and *Postia placenta* (Ide *et al*., 2012). These results suggest that, there may be a unique mechanism that activates the fungal secondary metabolic system during nitrogen limitations. Some restrictive media has also been reported like potato dextrose agar (PDA) or malt extract medium (ME) for restrictive growth of fungus (O'Callaghan *et al*., 2003).

P450 expression profile in *Candida albicans* and *Candida tropicalis*

Besides different types of molds, yeasts like *Candida albicans* and *C. tropicalis* show response to suitable media in expressing mRNA of CYP. In yeasts, less than 5% of nuclear genes have an intron (Bon *et al*., 2003). However, there is no intron in cytochrome P450 genes of *C. albicans* and *C. tropicalis* (http://p450.riceblast.snu.ac.kr/). In several previous studies, genomic DNA has been used as a template to amplify P450 genes (Craft *et al*., 2003; Kim *et al*., 2007). However, for the first time, we validated suitability of any liquid media that can be used to study expression profile of some P450 genes using these yeasts. For this, we cultured the yeasts in different media like PD broth, synthetic media under nitrogen-rich or nitrogen-limited conditions. The yeast cells were grown with shaking (200 rpm) at 30°C for 3 days. Then, total RNA was extracted using RNeasy Plant Mini Kit (QIAGEN). The RNA was treated with DNase I (Takara), and first-strand cDNAs were synthesized with QuantiTect Reverse Transcription Kit (QIAGEN) according to the instructions of the manufacturer. PCR amplification

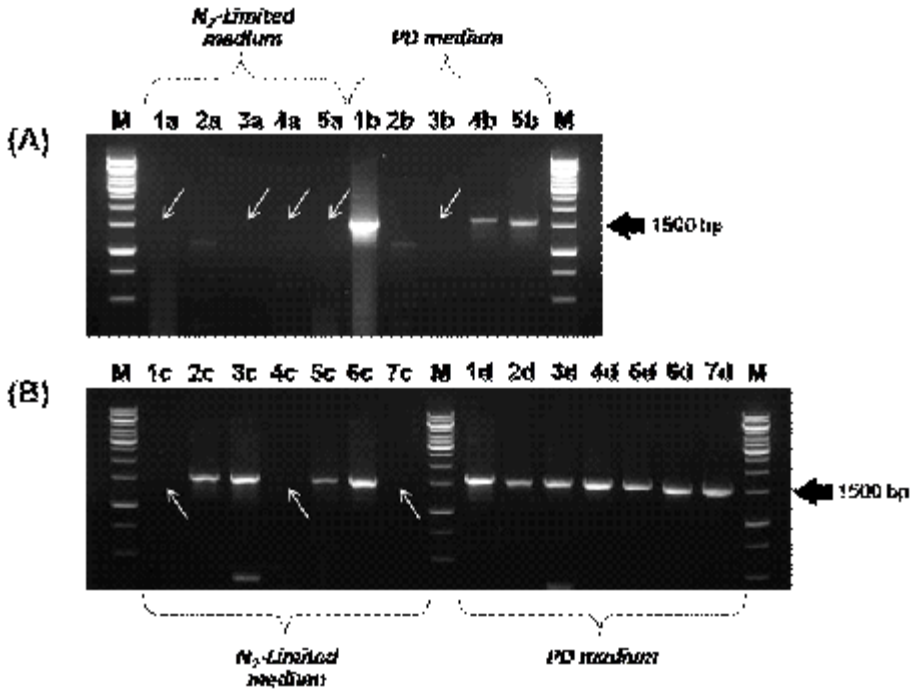

Fig. 1. Transcriptomics survery of CYP52 genes in *Candida albicans* and *C. tropicalis*. (A) Five genes of *C. albicans* are surveyed. (B) Seven genes from *C. tropicalis* are surveyed. Among five genes in *C. albicans*, four are expressed in Potato Dextrose (PD) broth, whereas only one was expressed in nitrogen-limited synthetic medium. Similarly, all genes of *C. tropicalis* were amplified from the cDNA made from total RNA extracted from the yeast cultured in PD broth. Some genes were not amplified from the cDNA made from nitrogen-limited medium, suggesting that PD broth is suitable for CYP expression in yeasts. Lanes 1a and 1b (CYP52A21), Lanes 2a and 2b (CYP52A22), Lanes 3a and 3b (CYP52A23), Lanes 4a and 4b (CYP52A24), Lanes 5a and 5b (CYP52C3), Lanes 1c and 1d (CYP52A1), Lanes 2c and 2d (CYP52A12), Lanes 3c and 3d (CYP52A14), Lanes 4c and 4d (CYP52A17), Lanes 5c and 5d (CYP52A20), Lanes 6c and 6d (CYP52B1), Lanes 7a and 7b (CYP52C1)

of CYP52 genes was carried out using gene-specific primers (Table 1). All the primers were purchased from the Cosmo Genetech Co, Ltd., South Korea. The PCR assay was performed in a total reaction volume of 50 µl consisting of first-strand cDNA solution (1 µL), dNTP (200 µM), primers (2 µM each), DMSO (2%), and Phusion DNA polymerase (0.02 U/µL) in Phusion HF buffer. The reaction conditions were programmed as follows: denaturation at 98°C for 2 min; 35 cycles of 98°C for 30 s, 55°C for 20 s, and 72°C for 60 s; and final extension at 72°C for 5 min. The PCR products were separated by 1.5% agarose gel electrophoresis and visualized on a UV-transilluminator.

In filamentous fungi, synthetic medium under nitrogen-limited condition was found to be suitable for expressing maximum P450s (Matsuzaki and Wariishi, 2004; Nazir *et al*., 2010; Ide *et al*., 2012). The nitrogen-limited medium exerts a stress condition to fungus that influences the fungi to express several genes particularly cytochrome P450 genes. In our experiments, ordinary media like PD broth was found to be suitable for the expression of CYP in *C. albicans* or *C. tropicalis*. The results are summarized in Fig. 1.

Supplementation of media and culture conditions (temperature, pH, incubation period) for P450 expression

Secondary metabolites are considered as the major influencing factors for expressing several genes. In an experiment with *Aspergillus fumigatus*, it was revealed that LaeA positively influenced the expression of 20-40% of major classes of secondary metabolite biosynthesis genes like P450 monooxygenases (Perrin *et al*., 2007). Nazir *et al*. (2010) studied the expression profile of P450s in *Aspergillus oryzae* using nitrogen-limited medium and found that considerable expression of different P450s starts after 5 days of incubation, and the expression could be detected by RT-PCR until 21 days, however maximum number of genes were expressed in a 10-day culture. A wide range of pH is suitable for expressing P450 indifferent fungi. A pH of 6.0 was found to be suitable for expressing P450s in *Aspergillus oryzae* (Nazir *et al*., 2010), *Postia placenta* (Ide *et al*., 2012) and *Phanerochaete chrysosporium* (Matsuzaki and Wariishi, 2004), whereas pH-4.5 was suitable in the case of *Aspergillus ochraceus* (O'Callaghan *et al*., 2006).

Thiamine on expression and maturation of P450

Thiamine has influence on both expression and maturation of several genes like *CYP* (Nazir *et al*., 2010), *NMT1* (Cheah *et al*., 2007), and *thiA* (Kubodera *et al*., 2003). Nazir *et al*. (2010) recovered total RNA from *Aspergillus oryzae* cells grown in synthetic medium with or without exogenous thiamine (0 or 1 mg/L), and found that CYP5076C1 was spliced to produce frame-shifted variants in *A. oryzae* when it was grown with additionally added thiamine. On the other hand, a mature variant was produced when it was grown without thiamine. Their functional expression might also be regulated by sophisticated maturation mechanisms at a post-transcriptional stage (Winkler *et al*., 2002; Cheah *et al*., 2002; Kubodera *et al*., 2003; Thore *et al*., 2006). In higher eukaryotes like human, CYP of liver was found to be expressed only in absence of thiamine (Yoo *et al*., 1990). Also, the effect of thiamin was clearly demonstrated in the filamentous fungus, *Neurospora crassa*, where *NMT1* mRNAs expression was enhanced in absence of thiamine (Cheah *et al*., 2007), In contrast, *NMT1* is repressed by excess thiamine in *N. crassa* (McColl *et al*., 2003). Supplementation of thiamine (vitamin B1) in growth medium reduced the expression of *A. oryzae thiA* mRNA (Kubodera *et al*., 2003), and this was done by adding riboswitching aptamer (TPP) with mRNA. Deletion of the TPP aptamers disrupted the responsiveness to thiamine. It is believed that thiamine is phosphorylated after entering cells to generate TPP, and the resulting coenzyme acts as a ligand in riboswitch-mediated controlling of RNA splicing in fungi (Sudarsan *et al*., 2003).

CONCLUSIONS

For isolation of P450 genes is the primary step for making a cDNA library, and expression of the genes in liquid media would be the crucial step. The understanding on expression profile of cytochrome P450 genes in both filamentous fungus and yeast has been focused in this mini-review. A clear study on expression profile of P450 in yeasts (*Candida albicans* and *C. tropicalis*) is reported here for the first time. Thus, the findings would widen the application of cytochrome P450s of fungi in industrial application.

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