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# ISOLATION AND CHARACTERIZATION OF LIGNIN PEROXIDASE FROM TROPICAL RESUPINATE WHITE ROT FUNGI

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ราฟอกขาวกลุ่มรีซุพพิเนทมีอยู่มากและพบได้ทั่วไปในเขตร้อน รากลุ่มนี้มีบทบาทสำคัญในการย่อยสลายไม้ในป่า แต่องค์ความรู้เกี่ยวกับลักษณะสมบัติของรากลุ่มนี้ในเขตร้อนยังมีอยู่น้อย งานวิจัยนี้ได้เก็บรวบรวมตัวอย่างรารีซุพิเนทบนขอนไม้ผุจาก 7 จังหวัด ในประเทศไทย รารีซุพพิเนททั้งสิ้น 25 ตัวอย่างสามารถถูกคัดแยกและจัดจำแนกได้ในระดับชนิด หรือสกุล และพบว่ารารีซุพพิเนทเขตร้อนเหล่านี้มีสมบัติที่เป็นเอกลักษณ์บางประการ เช่น การเป็นแหล่งของลิกนิน เพอร์ออกซิเดส ซึ่งเป็นเอนไซม์ที่พบได้น้อยในกลุ่มราฟอกขาวทั่วไป การทนทานต่ออุณหภูมิสูง และ การย่อยสลายลิกนินในเนื้อไม้ได้อย่างจำเพาะ จากตัวอย่างราทั้งหมดพบว่า *Phanerochaete sordida* Sk7 มีสมบัติที่เป็นเอกลักษณ์ดังกล่าว และสามารถย่อยสลายสารที่มีโครงสร้างแตกต่างกันได้อย่างมีประสิทธิภาพสูงสุด เมื่อนำ *P. sordida* Sk7 มาศึกษาเปรียบเทียบความสามารถในการย่อยสลายสีสังเคราะห์มาตรฐานที่มีความเป็นพิษและเสถียรสูง (Reactive black 5) กับราสายพันธ์มาตรฐาน (*Phanerochaete chrysosporium*) ภายใต้สภาวะทางกายภาพและเคมีที่กันพบว่า *P. sordida* Sk7 มีประสิทธิภาพในการย่อยสลายสีที่ดีกว่าราสายพันธ์มาตรฐานและ เสถียรต่อสภาวะการเปลี่ยนแปลงกรดเบส อุณหภูมิ และปริมาณสารเริ่มต้น กลไกการกำจัดสีของ *P. sordida* Sk7 ไม่ได้เกิดจากกลไกการดูดซับของเส้นใยรา แต่เกิดจากกลไกการย่อยสลายโดยการทำงานร่วมกันของแลคเคส และลิกนิน เพอร์ออกซิเดสเป็นหลัก การตรวจสอบผลที่ได้จากกระบวนการกำจัดสีโดย *P. sordida* Sk7 พบว่า ไม่มีสีหลงเหลือ อีกทั้ง และความเป็นพิษต่อพืช ซึ่งบ่งชี้ได้ว่าการกำจัดสีโดยราชนิดนี้เกิดขึ้นได้อย่างสมบูรณ์ ผลการศึกษาระบบเอนไซม์ของ *P. sordida* Sk7 พบว่า ลิกนิน เพอร์ออกซิเดสถูกผลิตขึ้นเป็นหลัก และมีบทบาทสำคัญในการบำบัดสีสังเคราะห์ดังกล่าว ลิกนิน เพอร์ออกซิเดสจาก *P. sordida* Sk7 ได้ถูกทำให้บริสุทธิ์ เพื่อศึกษาลักษณะสมบัติเบื้องต้น พบว่ามีความเสถียรต่อสภาวะกรดเบสที่ 3 ถึง 6 และอุณหภูมิที่ 10 ถึง 50 องศาเซลเซียส ลำดับเบสของยีนลิกนิน เพอร์ออกซิเดสจาก *P. sordida* Sk7 ได้ถูกศึกษาและเปรียบเทียบ พบว่ามีความคล้ายคลึงกับลำดับเบสของยีนลิกนิน เพอร์ออกซิเดส ไอโซไซม์ H8 ของ *P. chrysosporium*.

สาขาวิชา วิทยาศาสตร์ชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWORDS: DECOLORIZATION / LIGNIN PEROXIDASE / RESUPINATE FUNGI / WHITE ROT FUNGI

PONLADA PERMPORNSAKUL: ISOLATION AND CHARACTERIZATION OF LIGNIN PEROXIDASE FROM TROPICAL RESUPINATE WHITE ROT FUNGI. ADVISOR: ASSOC. PROF. HUNSA PUNNAPAYAK, Ph.D., CO-ADVISOR: PROF. DONALD KOBAYASHI, Ph.D., PROF. DOUGLAS EVELEIGH, Ph.D., 38 pp.

Resupinate white rot fungi are abundant throughout tropical regions. They play an important role on wood decaying in the forest, but little is known about their characteristics. In this study, tropical resupinate fungi were collected from dead-wood stumps in seven provinces of Thailand. Twenty-five specimens were successfully isolated and identified into specific or generic levels. Characterization study revealed several unique characters of these tropical resupinate white rot fungi including the potential source of lignin peroxidase which scarcely found in typical white rot fungi, thermotolerant ability, selective delignification property. Among these fungi, *Phanerochaete sordida* Sk7 showed a remarkable biodegradation characters by efficiently biodegrade structurally diverse substances. A comparative study on decolorization ability of a model azo dye, reactive black 5 under various physicochemical parameters between *P. sordida* Sk7 and the reference fungus, *Phanerochaete chrysosporium* suggested greater biodegradation ability of *P. sordida* Sk7 over a wide range of pHs, temperatures and dye concentrations. The decolorization mechanism of *P. sordida* Sk7 occurred through degradation activity of laccase and lignin peroxidase rather than mycelial biosorption. None of residual dye and phytotoxic natures of the dye were detected in its metabolites which indicated the successful treatment. Therefore, this fungus was purposed as a good candidate of white rot fungus for bioremediation and biopulping aspects. Since a great biodegradation ability of this fungus was proved, enzymatic degradation system of this fungus was considered. Lignin peroxidase was found to play significant role in its degradation system. Therefore, lignin peroxidase from this fungus was further purified and characterized and it was found to be stable over pH 3-6 and temperature ranging from 10 to 50 ˚C. A full-length of lignin peroxidase cDNA was obtained and found to be highly similar with LiP isosyme H8 from *P. chrysosporium*.



Field of □tudy: Biological Sciences
Academic Year: 2014

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....
Co-Advisor's Signature .....

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# CHAPTER I

# INTRODUCTION

## 1.1 Rationale

Lignin is the most recalcitrant natural polymer which provides strength and protective barrier within the woody cell wall by forming a complex with hemicelluloses to encapsulate cellulose against microbial attack (Gellerstedt and Henriksson 2008). A heterogeneous hydrophobic polymer of lignin make biodegradation studies a challenging task (Schmidt 2006). White rot fungi are the only organisms that can efficiently degrade and mineralize lignin by producing of extracellular lignin-modifying enzymes, mainly laccase, manganese peroxidase and lignin peroxidase (Hammel and Cullen 2008). These enzymes generate non-specific free radicals which are capable of breaking down a variety of structures of recalcitrant substances. Among lignin-modifying enzymes, lignin peroxidase (LiP; EC 1.11.1.14) contains the highest redox potential and plays a key role in lignin mineralization by initiating nonspecific chain degradation reaction on both phenolic and non-phenolic structures of lignin (Tien and Kirk 1984). LiP-producing fungi are known to be the rarest group among white rot fungi. However, researchers studying white rot fungi have found out that most of the potent LiP producers such as *Phanerochaete chrysosporium*, *P. sordida, Phlebia radiate, Irpex lacteus,* and *Bjerkandera adusta* share common flattened or effused fruiting bodies are classified into a group of resupinate white rot fungi (Mester and Field 1998, ten Have and Teunissen 2001, Hirai, Sugiura et al. 2005, Tanaka, Koike et al. 2009). The research field on employing white rot fungi for biological applications has also been highly expressed by the model of white rot fungi, *P. chrysosporium* which demonstrated an ability to mineralize lignin and structurally diverse pollutants (Bumpus, Tien et al. 1985, Singh and Chen 2008, Pavko 2011). The nonspecific degradation of lignin peroxidase has become of interest in bioremediation (Singh and Chen 2008).

Among the xenobiotic pollutants, dye wastewater is one of the most difficult to treat. The dyes are highly stable and hard to be degraded due to they contain diverse complex aromatic structures which were synthetic origin. They could be classified according to chemical structure into azo, anthraquinone, triphenylmethane, heterocyclic and polymeric structures (Robinson, McMullan et al. 2001). These dyes create ecological problems after being released into environment due to the presence in part though in very low concentrations, they are highly visible and can interfere photosynthetic activity of aquatic life (Nigam, McCallum et al. 2000). Particularly, azo dyes have been identified as major problematic compounds in textile wastewaters since they represent about one-half of all the dyes in common use, possess high toxicity and highly persistent in the environment

(Michaels and Lewis 1985). Although dyes wastewater treatment conventionally is achieved by physicochemical methods such as flotation, ozonation and adsorption, they have relatively high operating costs, limited applicability and low sustainability (Kaushik and Malik 2009). Biological methods have proven to be promising decontamination approaches, can be cost-effective of broad applicability and are ecological friendly. However, the limitations of using organism for the treatment include their ability to survive and maintain metabolism under a variety of environmental conditions, and the dye toxicity need to be considered. The tropical resupinate white rot fungi which are aggressive saprotrophs may have a tolerance to unusual than their temperate counterparts (Magan 2007). They may consider as bioresource for bioremediation program.

This study demonstrates the tropical resupinate white rot fungi as a bioresource and provides proof of concept for the bioremediation. New and more efficient organism need to be considered for this green technology. In this study, a number of tropical resupinate white rot fungi were collected from natural habitats in Thailand. They were isolated and identified based on morphological and also molecular biological characteristics. All isolates were screened for LiP production and characterized for growth, thermotolerant ability and lignocellulolytic enzyme production. The isolates which performed the most efficient in decolorization was selected to further evaluated on bioremediation potential by using reactive black 5 in comparison with *Phanerocheate chrysosporium*. Finally, the lignin peroxidase from this selected fungus was characterized including production, purification and nucleotide sequencing. Results from this research could provide valuable insight into fundamentals and progress to the practical application of resupinate white rot fungi for bioremediation.



### 1.2 Objectives of this study

1. To isolate the resupinate fungi that produce lignin peroxidase
2. To identify a selected fungus for producing the high yield of lignin peroxidase
3. To purify and characterize lignin peroxidase from the selected fungus
4. To evaluate biodegradation ability of selected fungus
5. To obtain the lignin peroxidase gene

### 1.3 Key words

Dye decolorization / Lignin peroxidase / Resupinate fungi / White rot fungi

### 1.4 Anticipated benefits

1. Characteristics of tropical resupinate white rot fungi in Thailand will be revealed.
2. An efficient lignin peroxidase-producing resupinate fungus will be obtained.

# CHAPTER II

# LITERATURE REVIEW

## 2.1 Composition of wood

Wood primarily consists of an association of polymers, generally termed lignocellulose. It is complex association of lignin with carbohydrate polysaccharides of cellulose and hemicelluloses (Figure 2.1).The general woody plant cell structure is illustrated in Figure 2.2. The middle lamella is the amorphous region between the cells and functions by binding the cells together. In the early stages of growth it is mainly composed of pectic substances, but eventually it becomes highly lignified (Webster and Weber 2007). The primary wall is a thin layer (0.1-0.2 microns thick) that consists of cellulose, hemicelluloses, pectin and protein and is completely embedded in lignin (Roberts 1996). The secondary wall is below the primary wall, and comprises nearly all of the cell wall. It is divided into three layers including primary wall, middle lamella and secondary wall (Fig. 2.2).

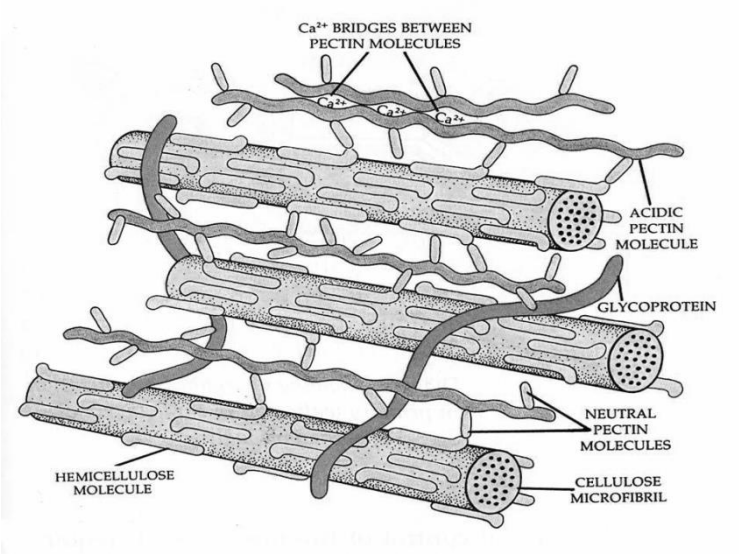



**Figure 2.1** A schematic presentation of lignin-carbohydrate complex of wood structure (https://www.studyblue.com).

Lignocellulose is comprised of 40–50% of cellulose, 20–35% of hemicelluloses and 15-35% of lignin (Pérez, Munoz-Dorado et al. 2002). Cellulose is a linear polymer of glucose subunits linked together by β-1,4-glucosidic bonds with the degree of polymerization up to 15,000. In wood, the cell wall cellulose forms microfibrils and fibers stabilized by hydrogen bonds between hydroxyl groups of the adjacent cellulose chains (Figure 2.2). Hemicelluloses are heteropolysaccharides, which are comprised of a β-1,4-linked polysaccharide backbone with different degree of substitution (Figure 2.2). The carboxyl groups of hemicellulose covalently bond with lignin via ether and benzyl ester linkages. Lignin is heterogeneous phenylpropanoid polymer including coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Figure 2.2). It occurs mainly in secondary wall, where lignin glues the adjacent cells together (Kuhad, Singh et al. 1997). It provides strength and resistance towards both mechanical and microbial attack to plant cell wall (Gellerstedt and Henriksson 2008). Lignin degradation is a rate-limiting step of carbon recycling since lignin forms an amorphious complex with hemicelluloses that encapsulates cellulose and combined with its hydrophobic nature of lignin yield breakdown system that is non-specific (Schmidt 2006).

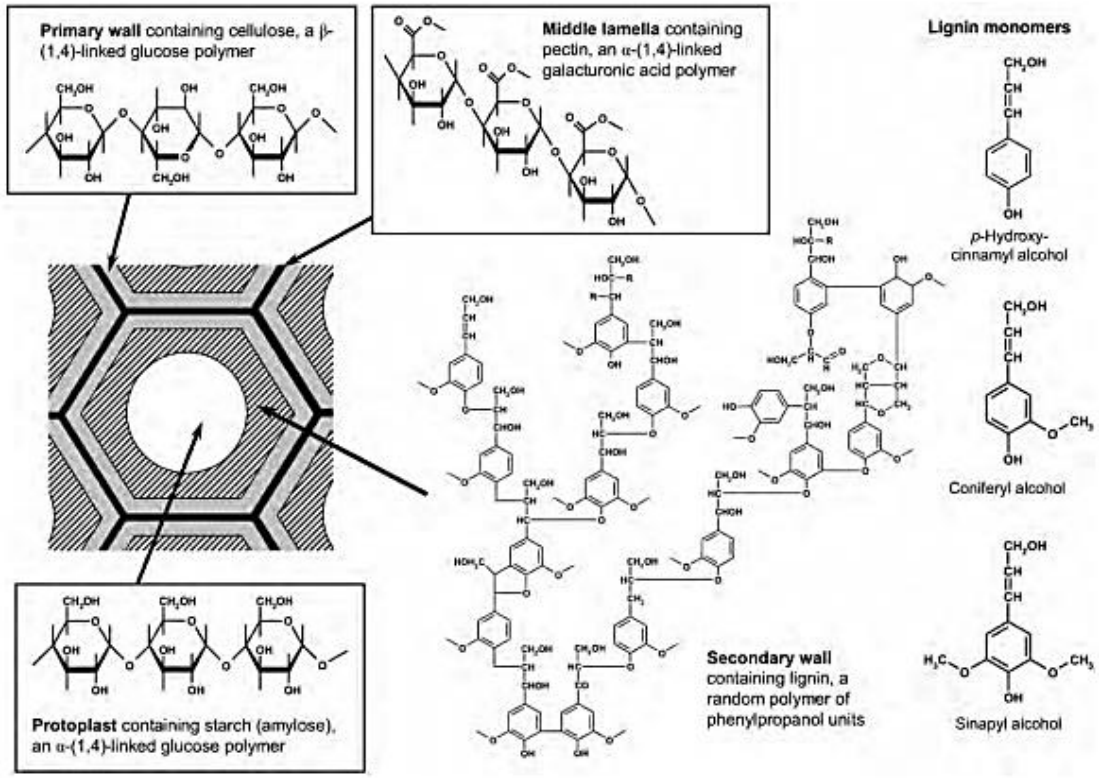



**Figure 2.2** Schematic presentation of carbon-containing polymers and their localization in woody tissue. Lignin consists of the three units (Webster and Weber 2007).

## 2.2. Wood degradation by fungi

Fungi are the primary degraders of wood due to mycelial structure allow filamentous fungi to penetrate substrate and transport nutrients from the holocellulose part of the lignocelluloses structure (Rabinovich, Bolobova et al. 2004, Sanchez 2009) and fungi are more resistant to natural inhibitors that limit bacterial growth such as tannins and phenolic compounds (Scheffer 1966, Hammel 1997). The many of wood-degrading fungi that have been emphasized to date are members of the Basidiomycota. Of the Basidiomycota there are two broad divisions, white rot and brown rot fungi. These names derive from the appearance of the wood due to the part of following degradation (Hammel and Cullen 2008). Brown rot fungi efficiently degrade wood polysaccharides leaving behind the brown lignin which is of poor strength (Blanchette 1995), whereas white rot fungi are able to degrade the whole wood components including cellulose, hemicellulose and lignin. Lignin degradation is a critical step for carbon recycling of wood. The complete mineralization of lignin has been reported only in basidiomyceteous white rot fungi (Kirk and Farrell 1987).

White rot fungi are so named because they leave a bleached appearance of the wood fiber after their attack (Guillén, Martínez et al. 2005). These fungi are distinctive in having extracellular non-specific mechanisms for the mineralization of lignin and lignin-like substances (Tuor, Winterhalter et al. 1995). Numerous fungi have been implicated in the biodegradation of lignin and the most extensive research has been on *Phanerochaete chrysosporium*, a model of white rot fungi (Kirk, Schultz et al. 1978, Bumpus and Aust 1987, Kersten and Cullen 2007). The bioremediation capability of *P. chrysosporium* has been demonstrated for a wide variety of xenobiotic pollutants (Asgher, Bhatti et al. 2008). There has been growing interest in studying the bioremediation ability of other white rot fungi, including *Bjerkandera adusta*, *Ceriporiopsis subvermispora*, *Irpex lacteus* and *Phlebia radiata* (Reddy 1995, Novotný, Erbanova et al. 2000, Robinson, McMullan et al. 2001, Eichlerova, Homolka et al. 2007, Tanaka, Koike et al. 2009, Babič, Likozar et al. 2012). However, white rot fungi vary in the manner they attack lignin, with some fungi preferentially removing lignin without a loss of cellulose. In selective decay, as found with *Ceriporiopsis subvermispora* and *Physisporinus rivulosus*, lignin and hemicellulose are degraded significantly more than cellulose (Enoki, Watanabe et al. 1999, Hakala, Lundell et al. 2005), whereas in non-selective decay for instance with *P. chrysosporium and Trametes vesicolor*, equal amounts of all lignocellulose components are degraded (Blanchette 1995, Tuomela, Vikman et al. 2000, Fackler, Schwanninger et al. 2007). The ability of white rot fungi to degrade lignin is due to the production of lignin-modifying enzymes. Physiological conditions for lignin degradation, as well as secretion patterns of the enzymes vary between different species (Hatakka 1994), as well as the enzyme properties including

thermostability and pH optimum (Eriksson, Blanchette et al. 1990, Hatakka 1994). Studies on the phylogeny and substrate preference of wood decaying fungi suggest that the decaying characteristics may be connected to the taxonomic position and ecology of the fungi. The evolution study of white rot fungi is under review (Hibbett and Thorn 2001, Binder, Hibbett et al. 2005). Resupinate fungi produce relatively simple, flattened, crust-like fruiting bodies with basidiospore formation on the top surface (Volk, 2000; Webster and Weber, 2007) which is considered a primitive characteristic(Hibbett and Thorn 2001). They are distributed throughout all major clades of Homobasidiomycetes and generally refer to corticoids and polypores groups (Hibbett and Binder 2002, Larsson, Larsson et al. 2004). They are comprised of over 500 species with 50 putative families (Binder, Hibbett et al. 2005). Though of worldwide distribution, currently they are but recorded mostly in temperate zone (Binder, Hibbett et al. 2005). The phylogenetic relationships among resupinate fungi are limited to the Polyporales and emerge as the least resolved clade in the Agaricomycotina (Larsson 2007). Identification of resupinate fungi is currently based on using classical morphology characterization (Wu, Mickley et al. 2007). However, their special basidiocarp characters including thin fruiting bodies with opened-pore on the top surface or sometime spore-bearing surfaces are readily contaminated and as a result can be difficult to isolate. Moreover, they can be easily overlooked due to their plain and flat fruiting bodies which are usually on the underside of the wood log (Wu 1990, Wu, Mickley et al. 2007) .

## 2.3 Lignin modifying enzymes (LMEs)

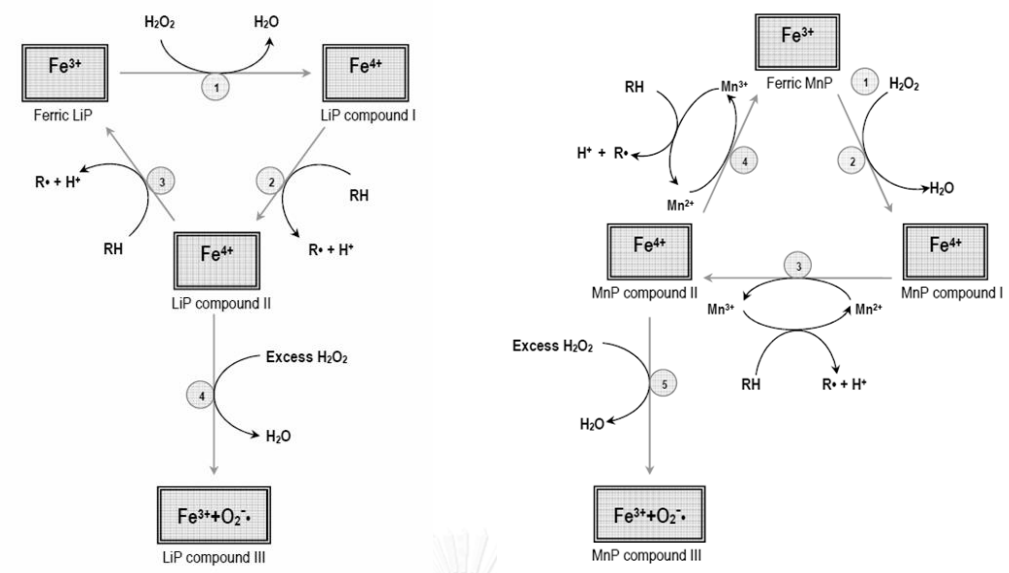
White rot fungi secrete one or more extracellular non-specific LMEs that are involved not only in lignin degradation but also degradation of several recalcitrant aromatic compounds including synthetic dyes, polycyclic Aromaic Hydrocarbon, plastic, biocides, explosives (Tišma, Zelić et al. 2010). They act by generating free radicals that randomly attack the lignin molecule, break covalent bonds and release a range of phenolic compounds which can be highly reactive but short-lived molecules (Mester and Field 1998). This is accomplished by catalyzing a one-electron oxidation, which results in the formation of radicals that undergo further spontaneous reactions (Kirk and Farrell 1987, Kluczek-Turpeinen, Maijala et al. 2007). Overall, degradation leads to the formation of water-soluble compounds and mineralization. In addition, the extracellular nature of the enzymes allows the fungi to access insoluble structure (Levin, Viale et al. 2003). White rot fungi and their enzymes are considered to be useful not only in some industrial processes like biopulping and biobreaching but also in bioremediation (Tišma, Zelić et al. 2010) . LMEs are produced during secondary metabolism (Wesenberg 2003) and the synthesis and secretion of LMEs is stimulated by nutrient availability. Nitrogen as a limiting nutrient can stimulate LMEs production in *P. chrysosporium* (Kirk and Farrell

1987). Conversely, synthesis of LMEs by some white rot fungi such as *Pleurotus osteaus* can be induced by nitrogen supplements (Freer and Detroy 1982). The three major LMEs are lignin peroxidase, manganese peroxidase and Laccase.

Lignin peroxidase (LiP, diarylpropane peroxidase, hydrogen-peroxide oxidoreductase, EC 1.11.1.14) was first discovered in *P. chrysosporium* (Tien and Kirk 1983) and named as "ligninases" due to their high redox potentials which enable the oxidation of dimeric lignin model compounds (Tien and Kirk 1983, Kuwahara, Glenn et al. 1984). LiP is able to degrade non-phenolic lignin units (up to 90% of the polymer). This enzyme is an extracellular hemeprotein, dependent of $H_2O_2$, with high redox potential and low optimum pH (Gold and Alic 1993). It shows little substrate specificity, reacting with a wide variety of lignin model compounds and even unrelated molecules (Barr and Aust 1994). LiP is a glycoprotein that contains the iron protoporphyrin IX (heme) group that is dependent on $H_2O_2$ for catalytic activity. LiP is expressed in multiple forms (isozymes) with MWs of 38-47 kDa (Table 2.1) (Fakoussa and Hofrichter 1999, Conesa, Punt et al. 2002). Lignin peroxidase was distinguishable from other peroxidases by the very low pH optima (near pH 3) and higher redox potentials (1.2 V at pH 3) enabling oxidation of non-phenolic aromatic substances, in contrast, manganese peroxidase has redox potential of 0.8 V at pH 4.5 (Cui and Dolphin 1991). LiP plays a role in initiation of nonspecific attack on both phenolic and non-phenolic structures of lignin and lignin-like aromatic model compounds (Tien and Kirk 1984, Wong 2009). The key reaction of the LiP is the one-electron-oxidation of non-phenolic structures that generates unstable aryl radical cations (Ander, Mishra et al. 1990, Schoemaker and Leisola 1990). The radical cations then act as oxidants to cleavage $C_\alpha$-$C_\beta$ bond in the side chain, $\beta$-O-4 bond between side chain and the aromatic ring (Eriksson, Blanchette et al. 1990). The catalytic cycle of LiP initially involves the oxidation of native Fe (III) enzyme by $H_2O_2$ to LiP compound I (Figure 2.3a). A one-electron reduction of compound I with an aromatic compound like veatryl alcohol results in the formation of compound II and a substrate radical. Compound II then undergoes another oxidation via a second aromatic substrate, whilst the free radical undergoes spontaneous reactions. In the presence of excess $H_2O_2$ compound II can be converted back to an inactive form of the enzyme LiP (compound III) (Figure 2.3a) (Wariishi and Gold 1990). These catalytic properties of LiP are of interest for applications in bioprocesses and bioremediation such as pulp and paper bleaching, bioethanol production from woody biomass, plastic recycling, and bioremediation such as polyaromatic hydrocarbons (PAH) and polychlorinated biphenyls (Bumpus and Aust 1987, Gusse, Miller et al. 2006, Singh and Chen 2008) However, the occurrence of LiP is uncommon since only some fungi are able to excrete LiP, however the fungi found to produce LiP are efficient lignin degraders including a resupinate fungi, *P. chrysosporium* (Tien and Kirk 1984), *Phlebia radiata* (Niku-Paavola, Karhunen et al. 1990), *Phlebia tremellosa*

(Vares, Niemenmaa et al. 1994), *B. adusta* (Heinfling, Martinez et al. 1998) also other polypores such a *Tametes versicolor* (Jönsson, Johansson et al. 1987) .

Manganese peroxidase (E.C.1.11.1.13) was first discovered in *P. chrysosporium* (Tien and Kirk, 1983). It is glycosylate protein and contains heme as the prosthetic group (Wariishi, Akileswaran et al. 1988). Like lignin peroxidase, manganese proxidase is also expressed in multiple forms with molecular weight ranging from 38 to 50 kDa (Table 2.1) (Fakoussa and Hofrichter 1999). The catalytic cycle of manganese proxidase is similar to that of lignin peroxidase and other peroxidases, but requires the presence of $Mn^{2+}$ to complete the cycle (Hofrichter 2002) (Figure 2.3b). A subsequent two-electron transfer from the heme is required to cleave the peroxide dioxygen bond and form manganese proxidase compound I. Subsequent reduction proceeds through manganese proxidase compound II. $Mn^{2+}$ ion is oxidised to $Mn^{3+}$ and acts as a one-electron donor to make compound II. The reduction of compound II occurs with the formation of another $Mn^{3+}$ from $Mn^{2+}$, consequently leading to the generation of native enzyme (Hofrichter 2002). High concentrations of $H_2O_2$ cause reversible inactivation of MnP and the formation of compound III ((Wariishi, Akileswaran et al. 1988). The presence of ligands such as malonate and oxalate assists in the stabilisation of $Mn^{3+}$ ions and promotes their release from the enzyme into the surrounding environment (Hofrichter 2002). The highly reactive $Mn^{3+}$ successively oxidises phenolic rings of lignin to unstable phenoxy radicals which further undergo spontaneous disintegration (Hofrichter 2002). The highly reactive phenoxy radicals are also involved in the cleavage of $C_\alpha$-$C_\beta$ bonds, and similarly alkyl-phenyl bonds, resulting in the formation of smaller intermediates including quinones and hydroxyquinones. Oxalic acid is another stabilizing chelator secreted by the fungi and acts as a diffusible redox-mediator, allowing manganese proxidase to oxidise and depolymerise the natural substrate lignin as well as recalcitrant xenobiotics and textile dyes (Heinfling, Martinez et al. 1998). In the absence of radical mediators, manganese proxidase mainly oxidizes phenolic lignin substructures, whereas in the presence of mediators, manganese proxidase oxidizes non-phenolic lignin substructures (Wariishi, Akileswaran et al. 1988). Manganese proxidase also catalyses the oxidation of several mono-aromatic phenols, including aromatic dyes. In contrast to LiP, MnPs are widespread among lignin-degrading fungi including both rot and litter-decomposing basidiomycetous species (Hatakka 1994, Lankinen, Hildén et al. 2005)




(a) (b)

**Figure 2.3.** Catalytic cycles of lignin peroxidase (a) and manganese peroxidase (b)(Gold, Wariishi et al. 1989).

Laccase (benzenediol oxygen oxidoreductase, EC 1.10.3.2) are known in plants, fungi and insects, where they play a variety roles including humification processes (Bourbonnais and Paice 1990) , pigment formation (Clutterbuck 1990), differentiation of sporulation and resting structures in basidiomycetes, formation of polyphenolic glue that binds hyphae together (Thurston 1994) , as well as fungal plant-pathogen/host interaction (Thurston, 1994). It is polyphenol oxidases that catalyze one-electron oxidations of phenolic compounds multicopper oxidases (Baldrian, 2006). The terminal electron acceptor in the catalytic reaction is molecular oxygen, which is reduced to water (Thurston 1994). The complete crystalline structure of laccase containing four copper atoms in the active site has been published from *Trametes versicolor* (Bertrand, Jolivalt et al. 2002). It catalyses the formation of phenoxyl radicals and their unspecific reactions leading to hydroxyl oxidation to ketone, alkyl-aryl–cleavage and demethoxylation in phenolic lignin substructures, as well as polymerization reactions (Youn, Kim et al. 1995). It is also able to oxidize also non-phenolic substructures of lignin in the presence of mediator (Call and Mücke 1997). Fungal laccase generally have molecular weight between 60-80 kDa (Table 2.1). Although laccase is commonly found in wood-degrading basidiomycetes, they have low redox potentials that only permit the oxidation of phenolic lignin units (often representing less than 10% of the polymer) (Hatakka 1994). To mineralize lignin, (Schlosser and Höfer 2002) suggested that white rot fungi need laccase that works synergistically with peroxidase through the formation of oxygen-mediated phenoxy radicals. These radicals present as

low molecular weight mediators which allow laccase to oxidise non-phenolic substrates (Bourbonnais and Paice 1990).

**Table 2.1** Properties of Lignin-modifying enzymes

| Enzymes | MW (kDa) | Optimal pH | Cofactor | Reaction | Reference |
|---|---|---|---|---|---|
| Laccase | 60-80 | 3.5-7 | $O_2$ | Phenols are oxidised to phenoxy radicals-mediator radicals | Thurston (1994) Hatakka et al. (2001) |
| Manganese peroxidase | 38-50 | 4-4.5 | $H_2O_2$ | Mn2+ are oxidised to Mn3+, Mn3+ oxidises aromatic substrates, oxidation of phenolic compounds to phenoxyl radicals that cleave Ca-Cb and alkyl aryl bonds | Gold and Alic (1993) Hofrichter (2002) |
| Lignin peroxidase | 38-47 | 2.5-3 | $H_2O_2$ | Abstracts an electron from the substrate aromatic ring, generating an aryl cation radical which decomposes by enzymatic and nonenzymatic processes | Gold and Alic (1993) Kirk and Farrell (1987) Fakoussa and Hofrichter, 1999 |

### 2.4 Biotechnological applications of white rot fungi

The extracellular non-specific nature of the catalysis by fungal lignin-modifying enzymes makes a diverse range of biotechnological applications including pulp and paper bleaching (Moreira, Feijoo et al. 2003), enhanced refining of wood chips or pulp (Young and Akhtar 1998). They also have a great potential on bioremediation of recalcitrant aromatic substances including chlorinated phenols, polychlorinated biphenyls (PCBs), DDT, dioxins, PAHs, alkyl halides, nitrotoluenes, chloroanilines and dyes (Pointing 2001, Wesenberg, Kyriakides et al. 2003, Asgher, Bhatti et al. 2008). Bioremediation research employing white rot fungi have been highly expressed (Bumpus, Tien et al. 1985, Singh and Chen 2008). The results illustrated that the efficient white rot fungi are mostly lignin peroxidase producers including *P. chrysosporium, P. radiate, B. adusta* and *T. vesicolor* since they capability to degrade a broad range of structurally diverse xenobiotics compounds (Wesenberg, Kyriakides et al. 2003, Regalado, García-Almendárez et al. 2004, Tišma, Zeliƈ et al. 2010).

#### 2.4.1 Biological treatment of synthetic dyes

Synthetic dyes are employed as coloring agents using in textile, paper, pharmaceutical, food, cosmetics, and pharmaceutical industries (Venkataraman 2012). They are chemically diverse

and can be broadly divided into azo, triphenylmethane or heterocyclic-polymeric structures (Venkataraman 2012). Even the presence of very low concentrations of dyes in effluent can be highly visible and undesirable (Nigam, McCallum et al. 2000). There are more than 100,000 commercially available dyes with over 700,000 ton of dyestuff produced annually (Hessel, Allegre et al. 2007). It is estimated that 10-14% of the dye (approximately 70,000 tons) is lost in the effluents during the dyeing process (Vijayaraghavan, Basha et al. 2013). Synthetic dyes may be resistant to biodegradation by indigenous micro-organisms and can persist in the environment for a long time, causing serious effects on ecosystems. Among synthetic dyes, azo dyes are the most widely used and disposal of waste water from industries (Robinson, McMullan et al. 2001). Beside the color problem, some dyes such as azo dyes also toxic to flora, fauna and humans. Azo dyes are characterised by the presence of one or more azo groups (–N=N–) (monoazo, diazo, triazo, polyazo) linked to phenyl and naphthyl radicals, which usually have some combinations of functional groups including amino ($–NH_2$), chlorine (–Cl), hydroxyl (–OH), methyl ($–CH_3$), nitro ($–NO_2$), sulphonic acid and sodium salts ($–SO_3Na$). They are designed to have high stability and resistance towards the oxidising agents (Reife 1993). The reduction of azo bonds yields amines which are highly toxic and carcinogenic in nature (Puvaneswari, Muthukrishnan et al. 2006). Since after being released into the aquatic environment, they may be converted into potentially toxic amines that impact the ecosystem, thus complete removal and/or mineralization of the azo from wastewater before being discharged into the environment is a matter of concern (Puvaneswari, Muthukrishnan et al. 2006). The current existing chemical and physical methods to remove synthetic dyes from effluents, e.g. adsorption, flotation, Fenton oxidation, reduction ($Na_2S_2O_4$), ion exchange, chlorination/ozonation and incineration, are rather costly, time-consuming, mostly ineffective and sometimes generate hazardous sub-products (Fu and Viraraghavan 2001, Robinson, McMullan et al. 2001). Thus, biodegradation can also play an important role in decolorization of the synthetic dyes due to the possibility to degrade and mineralize of recalcitrant and xenobiotic compounds. Microorganisms are sensitive to the presence of chemical substances and environmental conditions including dyes toxicity, pH, temperature, dye concentration and the presence or absence of oxygen (Ang, Zhao et al. 2005, Megharaj, Ramakrishnan et al. 2011). Fungi can be considered as suitable organism for the remediation purpose due to they have high surface-to-cell ratio characteristics of filaments that maximize both mechanical and enzymatic contact with the substrate (D'Annibale, Rosetto et al. 2006, Harms, Schlosser et al. 2011). The fungal mechanism for dye treatment and process optimization is important for designing the operational strategy. Three principal mechanisms are involved during the dye removal process mediated by fungi; biosorption, bioaccumulation and biodegradation. Biosorption is a metabolically independent process which involves the binding of solutes to the

fungal biomass and thus can occur in either living or dead biomass (Srinivasan and Viraraghavan 2010). Biodegradation is an energy intensive and metabolic dependent process, where the complex dye molecules are broken down into simpler molecules through the action of certain enzymes. Bioaccumulation is also energy and metabolically dependent process, where actively growing cells may accumulate the pollutants inside their cytoplasm (Chojnacka 2010).

Biosorption by using spent fungal biomass which is a by-product of metabolite production is a good and cheap source to be used in extensive use for dye biosorption (Fomina and Gadd 2014). Various functional groups, that are present on the fungal cell wall i.e. amino, carboxyl, thiol and phosphate groups, can bind dye molecules (Gadd 2009). Biosorption is a quick process which could get completed in a few hours. However, biosorption process is also affected by various parameters, such as pH, temperature, initial dye concentration and type of dye present in the solution which may resulting in temporary dye removal (Srinivasan and Viraraghavan 2010). Selection of a fungal strain for dye biosorption should be made by the capability to remove a wide variety of dyes belonging to different classes (Robinson, McMullan et al. 2001).

Biodegradation includes the breakdown of compounds which is mediated by the action of biological enzymes. Complete biodegradation is the total conversion of organic molecules into water, carbon dioxide and/or any other inorganic end products, ie. Mineralization (Reddy 1995, Wesenberg, Kyriakides et al. 2003). White-rot fungi secrete lignin-modifying enzymes that attack non-specifically to substrates and capable of degrading a wide variety of recalcitrant compounds and complex mixtures of pollutants, such as dyes (Wesenberg, Kyriakides et al. 2003). Biodegradation by white rot fungi depends on nutrient and fungal growth, thus initial dye concentration, pH, agitation, media components and presence of heavy metals should be considered (Moreira-Neto, Mussatto et al. 2013).

# CHAPTER III

# MATERIALS AND METHODS

## 3.1 Materials and Equipment

Autoclave: Ta Chang Medical Instrument Factory, Taipei, Taiwan

Autopipette: Pipetman, Gilson, Villiers, France

Barocycler (Pressure Biosciences, South Easton, MA)

Centrifuge, microcentrifuge: Model Denville 260D, Denville Scientific, NJ, USA

Cary 60 UV-Vis Spectrophotometer. Perkin Elmer, USA

DEAE Sephadex G-100 column, sigma-Aldrich, St. Louis, USA

Dialysis tubing cellulose membrane, Sigma-Aldrich, USA

Electrophoresis unit: Model mini-protein cell, Bio-Rad Applied Biosystem Company, Foster City, CA, USA

Incubator Shaker: New Brunswick Scientific Co., Edison, NJ, USA

Laminar flow: Model BV 123, ISSOC, Bangkok, Thailand

Nanodrop, Thermo Scientific, Waltham, Massachusetts, USA

Membrane filter: Whatman No.1, Tokyo, Japan

pH meter: Model PP-50, Sartorius, Goettingen, Germany

Shaker, Labcon, South Africa

UV-Vis Spectrophotometer, 2008 Unico, USA

Vivaflow50, MWCO 5000. Sartorius, Goettingen, Germany

Vivaflow50, MWCO 30000. Sartorius, Goettingen, Germany

Weight balance, 2 digits: Model BL610, Sartorius, Goettingen, Germany

## 3.2 Chemicals

ABTS (2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), SIGMA Chem. Co., St. Louis, MO

Acetic acid: Ajex Finechem, Auckland, New Zealand

Agarose: GenePure, Minneapolis, MN, USA

Ammonium sulfate: Ajex Finechem, Auckland, New Zealand

Astrazon Red, Dystar GmbH, Germany

Bovine serum albumin (BSA): Ajex Finechem, Auckland, New Zealand

Bromothymol Blue, Lachema, Czech Republic

Calcium chloride dihydrate: Ajex Finechem, Auckland, New Zealand

Chloroform: Carlo Erba, Milano, Italy

Congo red, Merck, Germany

Coomassie Brilliant Blue R-250, Sigma-Aldrich, St. Louis, MO

Copper sulfate: Carlo Erba, Milano, Italy

Deoxyribonucleotide triphosphate (dNTP): Vivantis, Shah Alam Selangor DE, Malaysia

Ethylene diamine tetra-acetic acid (EDTA): Ajex Finechem, Auckland, New Zealand

Hydrochloric acid: Carlo Erba, Milano, Italy

Isopropanol: Fisher Scientific, LE, UK

Lactophenol-cotton blue: Fluka, Buchs SG, Switzerland

Magnesium chloride: Ajex Finechem, Auckland, New Zealand

Manganese sulfate heptahydrate: Scharlau, Barcelona, Spain

Mercuric chloride: Scharlau, Barcelona, Spain

Methanol: Merck, Darmstadt, Germany

Methylene Blue, Merck, Germany

Potassium chloride: Ajex Finechem, Auckland, New Zealand

Potassium hydrogen sulphate: Ajex Finechem, Auckland, New Zealand

Procion Red Mx-5B, Sigma-Aldrich, USA

QIAquick PCR Purification Kit: QIAGEN, Inc., Valencia, CA, USA

Reactive Black 5, Sigma-Aldrich, USA

Reactive Brilliant Blue R, Sigma-Aldrich, USA

Sodium carbonate: Scharlau, Barcelona, Spain

Sodium chloride: Scharlau, Barcelona, Spain

Sodium dodecyl sulfate: Scharlau, Barcelona, Spain

Sodium thiosulfate: Merck, Darmstadt, Germany

Sodium hydroxide: Ajex Finechem, Auckland, New Zealand

Zinc sulfate heptahydrate: Scharlau, Barcelona, Spain

### 3.3 Collection and isolation of resupinate white rot fungi

Resupinate fungi together with evidence of lignin bleaching in wood were collected from 7 provinces in different parts of Thailand including Bangkok, Chonburi, Kanchanaburi, Nakhon Rachasrima, Nan and Roi-Ed (Figure 3.1). The wood containing a fungal fruiting body were cut into approximately 0.5 $cm^2$ pieces of and transferred to Petri dishes containing 2% malt extract agar (MEA; Becton-Dickinson, Baltimore, MD, USA) supplemented with 100 ppm of rose bengal and 100 ppm of chloramphenicol (Biolab Co., Ltd., Phra Nakhon Si Ayutthaya, Thailand) and 100 ppm of benomyl (Benlate, E.I. Dupont Nemours, Inc., Wilmington, DE; a fungicide with broad-spectrum activity toward ascomycetes). The cultures were incubated at 30 ˚C and monitored daily until white compact mycelia were developed. A small piece of agar containing fungal mycelia was transferred from the margin of the colony growing of each sample to Petri dish containing 2% MEA to obtain purified mycelia. Pure cultures were maintained on 1.5% MEA slants at 4 ˚C for further studies.

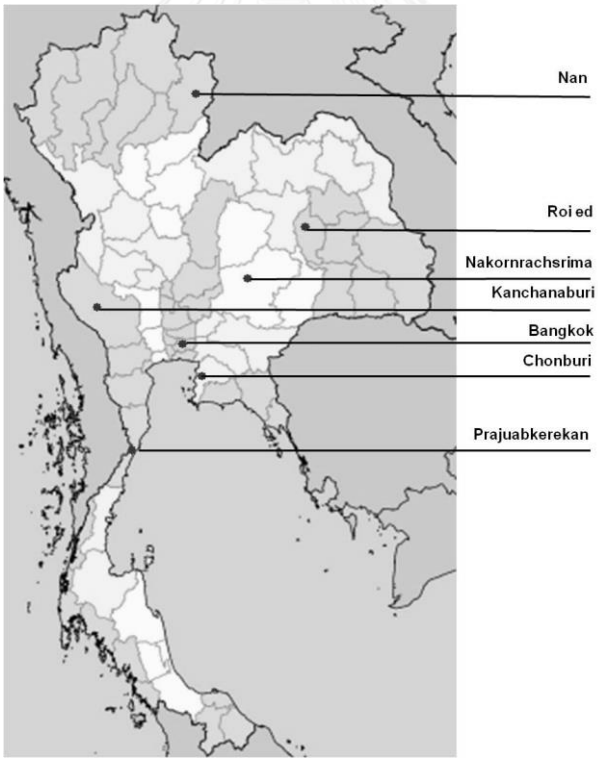



**Figure 3.1** Collecting sites of resupinate fungi in Thailand

### 3.4 Morphological observation

The fungal specimens were morphologically examined (Eriksson and Ryvarden 1975, Gilbertson and Ryvarden 1986, Wu 1990) . Macromorphological characteristics of hymeneal surface were observed from fresh specimens, whereas micromorphological characteristics were observed on dried specimens. Free-hand sections of the fruiting bodies were made under a dissected microscope with a razor blade. The dissecting portion was rehydrated by using 70% ethanol, chemical reactions in four mounting media included 5% (w/v) KOH, Cotton blue, Sulfoaldehyde and Melzer's reagent were observed. Microscopic structures including basidiospores, hyphae, cystidia were measured by an ocular micrometer. The fungal culture characteristics affected by different media including oat meal agar, potato dextrose agar and malt extract agar were also observed all at 1.5% (w/v).

### 3.5 DNA isolation, PCR amplification and nucleotide bases sequencing

Mycelia of the fungal isolates growing on the top of cellophane membranes placed on 1.5% MA plates were harvested 7 days after inoculation. Their genomic DNA was isolated by standard phenol-chloroform extraction (Davis 2012). The two genomic DNA regions, nuclear large-subunit ribosomal RNA (LSU) and the internal transcribed spacer (ITS), were amplified and partially sequenced using several primer combinations including LR0R-LR7 and internal primers, LR5, LR3R and LR3 for LSU and ITS4 and ITS5 for ITS (White, Bruns et al. 1990). Polymerase chain reactions were carried out using Chroma Taq DNA polymerase (Denville Scientific, Metuchen, NJ, USA). PCR mixtures were initially denatured at 94°C for 2 min, followed by 25 cycles of denaturation at 94°C for 10 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and quantified. Twenty nanograms of each PCR product was then sequenced by dideoxy chain termination method (GENEWIZ DNA Sequencing Service, NJ). Sequences were assembled and analyzed by using the DNAStar sequence analysis software (Lasergene).

### 3.6 Determination of thermotolerant ability

A culture plate was inoculated with one agar plug (5-mm diameter) followed by incubating at different temperatures including 30°C, 37°C and 43°C for 10 days. The fungal isolates that grew with colony diameter greater than 10 mm were scored for an average days of mycelium to cover the plate (Wu 1990).

### 3.7 Determination of enzymes production profiles

#### 3.7.1 Qualitative assay

Lignin modifying enzyme production was evaluated on Lignin modifying enzyme basal medium (LMB) supplemented with 0.2 % (w/v) glucose, specific substrate including ABTS or azure-B or phenol red and solidified with 1.6% (w/v) agar. 2% (w/v) MEA disc of actively growing mycelium was inoculated into each assay medium. 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS; 0.1%) medium plate was used to detect the ability to produce Lac (laccase) (Wolfenden and Willson 1982, Madhavi and Lele 2009). Formation of a green halo of oxidized ABTS around the fungal colony was determined as laccase positive. Phenol red (0.1%) medium plate was used to detect the ability to produce MnP (Orth, Royse et al. 1993). Change of phenol red color from yellow to red indicated the production of $Mn^{3+}$. Changes in the intensity of discoloration were recorded as 3 categories including yellow (+), orange (++) and red (+++). Azure B (0.01%) plates were used to detect LiP production (Archibald 1992). Decolorization of azure B (0.01%) indicated as the formation of a clear halo, was observed daily. Cellulases and xylanase production were evalulated on xylanolytic/cellulolytic basal medium (XBM/CBM) supplemented with 2% (w/v) xylan and 1% (w/v) carboxy methyl cellulose (CMC), respectively (Pointing 1999). Cultures 4 cm colony diameter were flooded with iodine and washed with $dH_2O$. A yellow halo around the colony indicated the degradation of xylan and CMC. All plate assays were carried out at 30°C and in triplicate

#### 3.7.2 Quantitative assay

Crude enzymes were prepared by growing each fungal isolate in 50 ml of lignin-modifying enzyme basal medium (LBM) (Tien and Kirk 1983). Five mycelial MA discs (5 mm in diameter) were inoculated into each flask and the cultures were incubated at 30°C in static condition for 7 days. Culture supernatants were taken (1 ml) for investigate the enzyme activities.

Lignin peroxidase: LiP (EC 1.11.1.14) activity was measured (Tien, Kirk et al. 1988) by monitoring the increase of the oxidation of veratryl alcohol to veratraldehyde at $A_{310}$. One unit (U) of LiP was defined as the amount of enzyme forming 1 µmol veratraldehyde min–1 ($\varepsilon$ = 9.3 $mM^{-1}$ $cm^{-1}$).

Manganese (II) peroxidase: MnP (EC 1.11.1.13) activity was assayed (Watanabe, Shirai et al. 2001) by monitoring the oxidation of 2,6 DMP at 469 nm ($\varepsilon$ = 27.5 $mM^{-1}$ $cm^{-1}$). One unit (U) of MnP activity is defined as the amount of enzyme to be required to oxidize 1 µmol of 2, 6 DMP in 1 min.

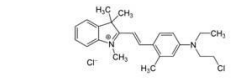
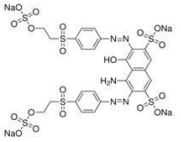
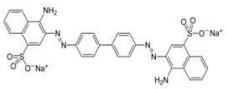
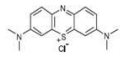
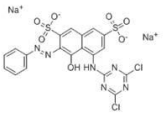
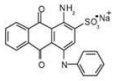
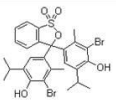
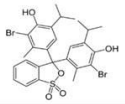
Laccase: Laccase (EC 1.10.3.2) activity was determined (Madhavi and Lele 2009) by using ABTS [2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) as substrate. Oxidation of ABTS was monitored at 30°C by measuring the increase in $A_{420}$ ($\varepsilon$ = 36.0 mM–1 cm–1). One unit (U) of laccase activity was defined as the amount of enzyme that oxidize 1 µmol of ABTS per minute (Wolfenden and Willson 1982).

For xylanase and cellulase assays, each fungal isolate was grown in 50 ml of basal medium supplemented with 2% (w/v) birch wood xylan and 1% (w/v) carboxymethyl cellulose (CMC), respectively (Tsiklauri, Khardziani et al. 1999). All cultures were incubated at 30°C and agitated constantly at 150 rpm for 7 days. Xylanase: $\beta$-Xylanase (a sort of hemicellulases) assay was carried out by Somogyi - Nelson reagent (Somogyi 1952), using D-xylose as a standard. The reaction mixture contained 0.1 ml of enzyme, 0.9 ml of 1% birch wood xylan (SIGMA) in 50 mM and sodium acetate buffer (pH 4.5) was incubated at 45 °C for 30 min. One unit of $\beta$-xylanase was defined as the amount of enzyme which liberates from xylan 1 µmol equivalent of xylose in one minute. The assay for $\beta$-(1 $\longrightarrow$ 4) D-glucanase activity was equal to that for xylanase except that the substrate was CMC (SIGMA), and D-glucose was used as a standard instead of xylose.

### 3.8 Determination of biodegradation ability

Eigh synthetic dyes were employed including astrazon red, bromothymol blue, congo red, coomassie blue, methylene blue, procion red, reactive black 5 and reactive blue (Table 2.2). They are representive of the dyes containing 5 different structures including monoazo, diazo, triazyne, anthraquinone and triphenylmethane. They were tested at 0.01% w/v. A single agar disc cut from the actively growing colony margin of cultures was inoculated in the agar plates. Cultures were incubated at 30°C for 10 days and decolorization of agar growth medium monitored. Each treatment was repeated in triplicate.

**Table 3.2** Synthetic dyes used in the decolormetric study.

| Class | Example | *C. I. generic Name | C. I. No. | Structure |
|---|---|---|---|---|
| Monoazo | Astrazon Red | Basic Red 46 | 48020 |  |
| Diazo | Reactive Black | Reactive Black 5 | 20505 |  |
| | Congo red | Direct Red 28 | 22120 |  |
| Triazyne | Methylene blue | Basic blue 26 | 92319 |  |
| | Procion Red | Reactive red 3 | 18159 |  |
| Anthraquinone | Reactive Brilliant Blue R | Reactive Blue 19 | 61200 |  |
| Triphenylmethane | Bromothymol blue | Acid blue 93 | 76595 |  |
| sriarylmethane | Coomasie Brilliant Blur R | Acid Blue 83 | 42660 |  |

## 3.9 Biological treatment of azo dye

### 3.9.1 Decolorization experiment

The decolorization was conducted in a 250-ml Erlenmeyer flask with 100 ml Malt Extract Broth (MEB) containing (g/l): 20 g malt extract, 0.5 g $KH_2PO_4$, 0.5 g $MgSO_4.7H_2O$ and 0.5 g $CaCl_2.2H_2O$ at pH 5.5 (Diwaniyan, Kharb et al. 2010). Each flask was inoculated with two agar plugs (8 mm diameter each) taken from the periphery of the 5-days-old colony grown on 2% MEA and incubated at 30°C under stationary conditions. After 3 days of fungal growth in MEB, the medium was supplemented with synthetic dyes at to a final condition of 100 mg/l. The time of subjecting the fungus to h dye was considered to be day zero and the absorbance of the dye in the medium was considered to be 100%. The fungal biomass was collected to study the effect of biosorption. The culture filtrate was centrifuged using refrigerated centrifuge at 10,000 rpm for 5 min (Eppendorf 5804 R). The percentage of RB5 decolorization was monitored daily. At intervals of every 24 h, 2 ml of culture medium was taken from the flask, centrifuged (Eppendorf 5804 R) and was read at 597 nm, UV-VIS-Spectrophotometer (Unico, Specord 2000, Jena, Germany).

### 3.9.2 Determination of the degree of decolorization

Decolorization activity was determined by measuring the decrease of the dye absorbance at its maximum visible wavelength at 597 nm. Medium without dye and inoculum was used as blank. Medium with dye plus without inoculum was used as control. All experiments were carried out in triplicates and the mean value was taken. The fungal biomass from each Erlenmeyer flask was taken after respective incubation period. It was crushed into a paste and suspended in 50 ml desorption solution, DS (ethanol: water 1:1) and centrifuged (12,000×g; 20 min; 30°C). The color of the CS and DS was read at maximum absorbance spectra of the dyes 597 nm for RB5, The uninoculated dye-containing medium was used as control. The decolorization efficiency was determined using the following equation (López, Guisado et al. 2006).

Decolourization (%) = [(Initial Absorbance – Final Absorbance) / Initial Absorbance] × 100.

Where final absorbance = (color absorbance of CS - color absorbance of DS)

3.9.3 Statistical analysis

All the experiments and analyses were run in triplicate and the data on optimization of Lignin peroxidase production, purification and characterization were subjected to statistical analysis using Analysis of Variance (ANOVA) (Miller Jr 1997) and Tukey test (Pizarro, Guerrero et al. 2002). In tables, the data values have been presented as mean ± standard error of three replicates and in figures the standard error Tukey test represent the significant differences of the mean values of original results of lignin peroxidase activites by alphabetic letters in the same table.

3.9.4 The effect of physical parameters on the decolorization

A variety of parameters affecting the decolorization including dye concentration (100 and 200 mg/l), pH (6, 7 and 8), temperature (25, 30, 35 °C) and agitation (0, 150 rpm). The experiment was monitored as mentioned above at 0, 1, 2, 3 and 4 day.

3.9.5 Biodegradation analysis

A. UV-vis spectral analysis

The supernatants of control and experimental samples were evaluated for decolorization by UV-vis spectral analysis. Spectrum analysis was carried out by scanning of the wavelength between 400 to 800 nm using Unico spectrophotometer.

B. HPLC analysis of degraded products

The degradation analysis was performed at RB5 concentration of 200 mg/l (Wu, Liu et al. 2007). Ten milliliters of samples were taken, centrifuged at 10,000 rpm for 30 min and filtered through 0.45 μm membrane filters (Millipore). The filtrate was extracted three times with methylene chloride and evaporated in rotary vacuum evaporator at 45 – 50 °C in water bath, after that the residue was dissolved in 2 ml methanol. Compounds were also extracted from control dye using the same method. The extract was dissolved in 5 ml of methanol and subjected to HPLC (Lichospher, Germany) analysis. Extracted samples were eluted using a C18 reversed phase column (RP-C18 phenomenex). The samples were analyzed using a gradient method with the 0.025 M phosphate buffer (pH =3.0) and acetonitrile mobile phase was employed in separation (Zhao and Hardin 2007). The injection volume was 100. The flow rate of mobile phase was 0.5 ml/min, and the UV-VIS detector was set at 285 nm. The analyses were carried out in duplicate.

3.9.6 Toxicity study

Phytotoxicity test was performed to determine the toxicity of the dye degradation products by using azo dye sensitive plants (Araújo and Monteiro 2005). Pesticide free seeds including sorghum (*Sorghum bicolor* Linn.), corn (*Zea mays* Linn.) and red kidney bean (*Phaseolus vulgaris* Linn.) were employed and allowed to germinate for 7 d. Their germination percentages and seedling shoot and root lengths were measured. Five replicates of three sets of samples including one soaked with water, second with the RB5 (200 mg/l), and third with the biodegraded RB5 which proved by HPLC were conducted.

## 3.10 Purification and characterization of Lignin peroxidase

3.10.1 Lignin peroxidase assay (see in Appendix C)

Lignin peroxidase was assayed by the method of Tien and Kirk, (1983). The assay involves the rate of formation of veratraldehyde as a result of oxidation of veratryl alcohol by the action of LiP in 100mM sodium tartarate buffer of pH 3 in the presence of $H_2O_2$.

3.10.2 Determination of protein content

During enzyme purification, the protein content of the samples was estimated (Bradford 1976) using Bovine Serum Albumin (BSA) as standard. BSA concentration of up to 1mg $ml^{-1}$ was used as standard stock solution and 100 μl of BSA stock solution was added to 900 μl of distilled water.



3.10.3 Preparation of crude enzyme

Production of LiP was carried out in 1 L Erlenmeyer flasks under 200 ml of manganese free-Kirk's medium (Kirk, Schultz et al. 1978) (Appendix A), sterilized and inoculated with 3-day olds of cellophane preparing fungal mycelium under sterile conditions. The fungal cultures were incubated at 30°C under static condition for 5 days. LiP was recovered by filtered through Whatman No.1 filter paper. The filtrates were centrifuged at 5,000 × g for 10 min and clear supernatants were pooled as crude extract for LiP assay and purification studies.

3.10.4 Purification step of LiP enzyme

Crude extract of LiP produced by *P. sordida* PBU 0058 The supernatant was further purified by the following steps.

3.10.4.1. Precipitation with ammonium sulfate and dialysis

The supernatant was brought to achieve 45-85% saturation at 4°C by gradual addition of crystals of ammonium sulfate. The mixture contents were thoroughly mixed and kept for overnight at 4°C. The resulting precipitate from each saturated solution was collected by centrifugation at 3,000 × g for 15 min at 4°C. After centrifugation the pellets were dissolved in minimal volume of 100 mM tartarate buffer pH 3 and dialyzed against the same buffer to remove ammonium sulfate for 72 hours with three changes of cold buffer. Total protein contents and LiP activity were determined before and after ammonium sulphate precipitation and dialysis. The dialyzate was concentrated by ultrafiltration.

3.11.4.2. Ion-exchange Chromatography

The partially purified enzyme obtained from ammonium sulfate precipitation dialysis was loaded at 300 µL/run on DEAE ion-exchange column (20 × 2.0) and 50 mM tartrate buffer pH 3 was used as elution buffer with the flow rate of 1ml/min. The fractions were collected and assayed for protein content and LiP activity. The LiP positive fractions were pooled together and dialyzed against the same buffer. The dialyzed positive fractions were collected and stored at -20 °C.

3.11.5 Molecular weight determination by SDS-PAGE



The purified LiP was run on native polyacryl amide gel electrophoresis (PAGE) and sodium dodecyl sulphate PAGE (SDS-PAGE) for estimation of its molecular weight (Neville 1971). The samples of LiP enzyme were subjected to SDS-PAGE on 10% poly acryl amide gels (Laemmli 1970). The approximate molecular mass of LiP was determined by calibration against broad range low molecular weight (21-116 kDa) markers (β- galactosidase, 116 kDA; phosphorylase B, 97 kDa; albumin, 66 kDa; ovalbumin, 45 kDa: carbonic anhydrase, 30 kDa and trypsin inhibitor, 21 kDa).

3.11.6 Characterization of purified LiP

The purified LiP enzyme was subjected to characterization through kinetic studies by studying the following

3.11.6.1 Effect of pH on enzyme activity

The LiP was incubated in difference pH buffers ranging from pH 2-8 (tartrate buffer pH 2, 3 malonate buffer 4 pH, tatrate buffer 5 pH, phosphate buffer 6, 7 pH, Tris buffer 8 pH, respectively) followed by standard LiP assay protocols. For study pH stability, the enzyme was incubated at different pH for 30 min at 4$^{o}$C prior to measure the residual activity.

3.11.6.2 Effect of temperature on Lip enzyme activity and stability

The LiP was incubated in difference temperatures in triplicate ranging from 25-100 $^{o}$C at pH 4.5 for 30 min before LiP assay. For study temperature stability, the enzyme was first incubated without substrate at different temperature for 30 min prior to measure in the residual activity.

3.11.6.3 Effect of substrate concentration

$K_m$ and $V_{max}$ of LiP activity was determined using a range of concentrations of veratryl alcohol (1-9 mM) as assay substrate, while keeping the enzyme concentration constant. The Michaelis Mention constant (Km) and maximum catalytic activity ($V_{max}$) was determined by constructing reciprocal plots. Simple Michaelis Menten graphs were drawn by plotting [S] against initial reaction rate (Vo) and the reciprocal Lineweaver-Burk plots were constructed by plotting [1/S] against 1/Vo to determine the accurate values of KM and Vmax (Schnell and Maini 2003).

3.11.6.4 Effect of metal ions

Effect of EDTA and metal ions such as $Ca^{2+}$, $Cu^{2+}$, $Fe^{2+}$, $Hg^{2+}$, $Mg^{2+}$, $Mn^{2+}$ and $Zn^{2+}$ were studied in concentrations of 0.1, 1.0 and 10 mM.

## 3.12 Construction of the full-length LiP cDNA

### 3.12.1 Design nested primers

The partial *lip* gene was amplified using the specific primers. The partial *lip* cDNA sequence was used to design a further set of primers for RACE PCR. RACE PCR products will be sequenced and used to design a pair of primers in order to obtain full-length *lip* cDNA. The full-length lip cDNA will be cloned into pGEM-T easy vector and correct DNA sequence will be confirmed by sequencing.

### 3.12.2 Isolation of RNA and DNase treatment

The frozen mycelial tissue obtained from the culture of fungus growing by was homogenized in TRIZOL REAGENT (SIGMA-ALDRICH) with Barocycler machine and centrifuged at 12,000xg for 10 min, then phase separated by the addition of chloroform. The nucleic acid phase was precipitated with isopropanol, washed with 70% ethanol, and solubilized in nuclease treated water. The RNA solution was treated with DNase using TURBO DNA-free protocol (Ambion, Woodland, TX) and quantified by nanodrop machine.

### 3.12.3 Synthesis of full length lip cDNA by RACE PCR (Figure 3.3)

Total RNA was extracted with TRIzol reagent (Life Technologies, Gaithersburg, Md.) and reverse transcribed according to the instructions of a First Choice RLM-RACE kit (Ambion, Inc., Austin, TX.). The 5′ and 3′ ends of the *lip* cDNA were amplified (Scotto–Lavino, Du et al. 2006) in two separate nested PCRs by using two gene-specific primers which designed based on lip conserve region and two adapter ligated primers into the cDNA. Primer sets designed on results of 3'-RACE and 5'RACE were used to clone full-length lip cDNA. The resulting PCR products were ligated into the PGEM-T Easy vector (Promega, Madison, WI) and transformed into *Escherichia coli* DH5$\alpha$ for sequencing.

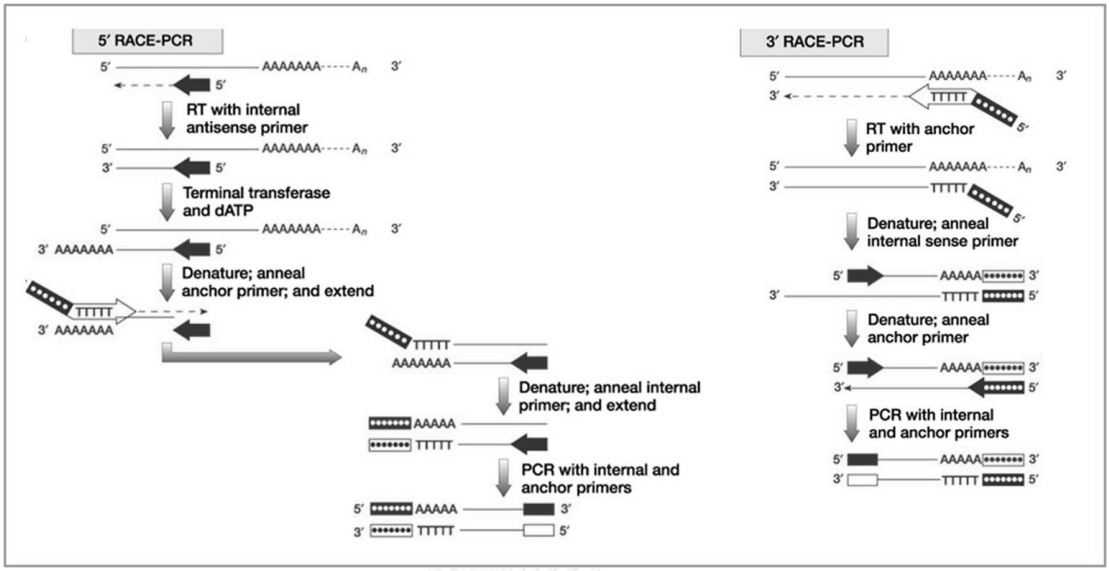



**Figure 3.3** Schematic of RACE-PCR method (Scotto–Lavino, Du et al. 2006)

3.12.4 LiP sequence comparison

The full-length lip cDNA was compared against lignin peroxidase nucleotide and protein sequences in the online databases using BLAST programs. Alignments of sequences will be made using CLUSTAL W multiple sequence alignment program (Thompson, Higgins et al. 1994).

# CHAPTER IV

# RESULTS

## 4.1 Collection and isolation of resupinate white rot fungi

Resupinate white rot fungi were collected from different area of Thailand (Table 4.1). Twenty-five resupinate white rot fungi were successfully isolated from 53 basidiocarp specimens on logs, stumps and branches (Figure 4.1).

**Table 4.1** Collection of resupianate fungi from Thailand.

| Collecting sites | Date | Samples | Isolates | Specimens No. |
|---|---|---|---|---|
| Bangkok | 30/04/09 | 4 | 1 | 13 |
| Chonburi | 04/05/09 | 9 | 5 | 3, 10, 16, 20, 23 |
| Kanchanaburi | 18/05/09 | 14 | 6 | 4, 9, 15, 17, 18 |
| Nakhon Ratchasima | 11/06/09 | 12 | 5 | 2, 5, 7, 14, 1, 6 |
| Roi Et | 18/08/09 | 3 | 2 | 1, 24 |
| Prachuap Khiri Khan | 02/01/09 | 3 | 2 | 8, 11 |
| Nan | 21/10/09 | 8 | 4 | 12, 19, 22, 25 |


Figure 4.1 Resupinate fungi specimens from Thailand

## 4.2 Mophological identification of resupinate white rot fungi

Twenty-five resupinate fungi were morphologically identified into 12 species or genera levels. Taxonomic descriptions of 12 identified species were provided as followed.

*Bjerkandera adusta* (Fr.) P. Karst.

Specimens examined: THAILAND, Nan Province. Doi Phuka, October 2008, collector P. Permpornsakul, Figure 4.1 (22)

Basidiocarp annual, resupinate, effused-reflexed to pileate, often with imbricate; pilei up to 3-5cm wide, up to 4 mm thick at the base, soft and pliable when fresh, hard and brittle when dry; pilear surface white to cream, becoming greyish to blackish azonate first finely velutious, later smooth to finely scrupose; pore surface grey to black, pores round to angular, 4-6 per mm, tubes grey to black, up to 2 mm long; context white and fibrous, up to 3 mm thick at the base. Hyphal system monomitic; generative hyphae with clamps, hyaline with small to large conspicuous clamps, moderately branched, 2-8 µm wide. Basidiospores oblong-ellipsoid, 4-5.5 × 2.5-3 µm.

*Ceriporia cystidiata* (Ryv. & Iturr.)

Specimens examined: THAILAND, Nakhon Ratchasima Province. Sakaerach, June 2008, collector P. Permpornsakul, Figure 4.1 (7)

Basidiocarps annual, resupinate, brittle when dry, 1 mm thick, pore surface white to pale cream, pores thin-walled, round to angular, slightly irregular, 6–8 per mm, tube layer concolorous to pore surface, up to 1 mm deep. Subiculum very thin, cottony, white. Hyphal system monomitic; generative hyphae hyaline, Thin- to slightly thick-walled, simple-septate, with sparse branching, negative in Melzer's reagent, 3–8 mm in diam. Basidia clavate, 4-sterigmate, 10–12 x 3.5–4.5 µm, simple-septate at the base. Cystidia present in the hymenium, tubular, thin-walled, with a slight apical encrustation, up to 90 µm long, 5–12 µm wide. Spores allantoid, hyaline, thin-walled, smooth, IKI-, 4–4.5 x 1 µm.

*Irpex lacteus* (Fr.) Fr., Elench.

Specimens examined: THAILAND, Kanchanaburi Province. Arawan National Park, May 2008, collector P. Permpornsakul. Figure 4.1 (4)

Basidiocarps effuse-reflexed, semi-pileate, also resupinate, pilei 10-20 mm across and extending 5-20 mm from the substrate, upper surface tomentose-pilose, cream-colored, whitish, to dingy yellow, margin sharp, somewhat incurved, in resupinate forms fringed to distinctly bounded. Hymenophora irpicoid, irregularly pore toward the margin, pores the angular, teeth up to 0.5 mm long. Whitish to orcharish, contex or subiculum 0.5-1 mm thick and concolorous. Consistency leathery, fibrous. The fungus has a tendency to form bracketlike pilei in rows. Hyphal system dimitic, generative hyphae thin to thick, 2-3 µm across, septa without clamps; skeletal hypha thick wall skeletoid hyphae (like lamprocystedia) exserted beyond the hymenium, thick-walled, incrusted part cylindrical to conical, 25-35 x 3-7 µm. Basidia cylindric-clavate, 20-25 x 3-4 µm , with 4 sterigmata, without basal clamp. Spores cylindric-ellipsoid, smooth, hyaline, 5.0-6.5 x 2.2-2.8 µm.

*Junghuhnia nitida* (Fr.) Ryv.

Specimens examined: THAILAND, Kanchanaburi Province. Arawan National Park, May 2008, collector P. Permpornsakul, Figure 4.1(17)

Basidiocarps resupinate, effuse, appressed closely to the substrate except when old at the edge, 2-3 mm thick, pinkish orchraceous with a pale yellowish or whitish margin; pores round or angular, 5-7 per mm, or sometime elongated and larger, margin distinctly bounded. Consistency soft when fresh, hard and brittle when dry. Hyphal system dimitic, generative hyphae 1.5-2.5 µm across, septa with clamps. Skeletocystidia thick-walled, numerous, rising out of the hymenium and some exserted, up to 200 µm long, upper half thick-walled, heavily encrusted, 8-12 wide, tapered towards base which bears a clamp. Basidia clavate, 11-15 x 4.0-5.5 µm, with 4 sterigmata and basal clamp. Spores ellipsoid to oval, smooth, hyaline, with drops, 4.0-4.5 x 2.0-3.5 µm.

*Macrohyporia dictyophora* (Cke) Johan, et Ryv.

Specimens examined: THAILAND, Kanchanaburi Province. Arawan National Park, May 2008, collector P. Permpornsakul, Figure 4.1 (18)

Basidiocarp resupinate-adnate, at first punctiform, then coalesced to pieces, up to 35cm long, 2-3 cm broad, about 0.5 mm thick. Pore surface white to sordid white, drying sordid tawny; margin thin,

white, velutinate; pores round, 3-4 per mm. Context white, very thin. Hyphal system dimitic: generative hyphae generally 2-3 µm thick, main stem up to 6 µm, colourless, branched, septate and without clamp-connections; binding hyphae somewhat inflated, outer wall curved and sinuose, nearly solid, ramuli 1.5-2 µm thick, main stem up to 6 µm thick, colourless. Cystidia absent. Basidia clavate, 12-14 x 3.5-4 µm, 4-spored. Spores globose to subglobose, 4.5-6 µm in diam., hyaline.

*Pachykytospora alabamae* (Berk. & Cooke) Ryv.

Specimens examined: THAILAND, Prachuab khiri kan Province. Huahin, January 2009, collector P. Permpornsakul, Figure 4.1 (8, 11)

Basidiocarp annual or reviving, resupinate, up to 2 mm thick, fleshy-rubbery when fresh, more leathery to corky on drying. Pore surface cream, orchraceous to pale brown, dull, pores round to angular, 4-5 per mm, dissepiments thin and entire, tubes up to 1 mm, margin sterile, often paler than the pore surface. Context pale brown, less than 1mm thick, spongy-fibrous, continuing continuing without change into the dissepiments. Hyphal system trimitic: generative hyphae thin-walled and hyaline, with clamps, mostly 2-3 µm wide, skeletal hyphae thick-walled, yellowish, 3-5 µm wide, binding hyphae abundant, thick-walled arboriform, the branches are often broken and difficult to distinguish between skeletal or binding hyphae. Cystidia absent. Spores oblong-ellipsoid, hyaline, thick-walled ornamented with longitudinal striae staining strongly in cotton blue, 9-12 x 4-6 µm, hyaline.

*Peniophora boidinii* (Reid)



Specimens examined: THAILAND, Nan Province. Doi Phuka, October 2008, collector P. Permpornsakul, Figure 4.1 (25)

Basidiocarp resupinate-effused, up to 0.13 mm thick. Surface smooth, cream with rosy tint, firmly attached to substrate. Hyphal system monomitic with clamps. Thin- to slightly thick-walled, 2-4 µm wide. No horizontal hyphal layer. Dendrohyphidia present. Gloeocystidia thick-walled, encrusted, 20-40 x 7-10 µm. Basidia narrowly clavate, 25-35 x 4.5-6.0 µm with 4-sterigmata. Basidiospores ellipsoid, 6-7 x 3-4 µm, thin-walled, smooth, hyaline.

*Perenniporia corticola* (Corner) C. Decock.

Specimen examined: THAILAND, Kanchanaburi Province. Arawan National Park, May 2008, collector P. Permpornsakul. Figure 4.1 (15)

Basidiocarps perennial, resupinate, effused, adnate, up to 15 mm thick, rather soft when fresh, chalky when dry. Hymenial surface cream to bright yellow, poroid, rarely cracked; margin thick. Pores round, 8-9 per mm. Hyphal system dimitic; generative hyphae, hyaline, septa with clamped. Extremely reduced arboriform skeletobinding hyphae, developing a much branched, up to almost coralloid-apical part in the dissepiments, densely intricate with dendrohyphidia-like elements are variebly observed in the dissepiments. Spores ellipsoid, apically distinctly truncate, thick-walled, with an apical germ pore, smooth, dextrinoid, cyanophilous, 4.6-5.1 x 3.2-4 µm.

*Perenniporia medulla-panis* (Jacq.Fr.) Donk.

Specimen examined: THAILAND, Chonburi Province. Kaokeaw, May 2008, collector P. Permpornsakul, Figure 4.1 (20)

Basidiocarp resupinate, adnate. Context fairly thin, up to 150 mm thick in section. Hymenial surface cream-colored, poroid, rarely cracked; margin thinning or rather determinate, usually concolorous. Pores ± angular or round, 5-7 per mm; tubes up to 1 mm deep. Hyphal system trimitic; generative hyphae nodose-septate. Context fairly uniform, composed of medullary layer, with fairly loose texture. Contextual generative hyphae colorless, 1.5-2.5 mm diam, thin-walled; skeletal hyphae dominant in context, colorless or yellowish, 2-3.5 mm diam, dichotomously branched, dextrinoid. Trama with fairly dense texture. Subhymenium not thickening. Cystidioles present, fusoid, 12-18 × 5-8 mm, thin-walled. Basidia clavate, 14-20 × 7-8 mm, 4-sterigmate. Spores ellipsoid-truncate, 4.5-6 × 4-4.5 mm, hyaline, thick walls, dextrinoid.



*Perenniporia subacida* (Peck) Donk.

Specimen examined: THAILAND, Kanchanaburi Province. Arawan National Park, May 2008, coll P. Permpornsakul, Figure 4.1(9)

Basidiocarps annual to perennial, resupinate, widely effused, up to 15 mm thick, rather soft when fresh, brittle and slightly coriaceous to hard when dry, on oblique substrates with sloping sterile and smooth areas between fertile areas. Pore surface cream to straw-colored, shiny when turned in incident light, margin narrow and light cream pores thin-walled, round to angular, 4-6 per mm, tubes concolorous with pore surface, tubes non-stratified or stratified, up to 4 mm deep in each layer, subiculum pure white and cottony. Hyphal system trimitic, generative hyphae hyaline and clamped, 3-5 µm wide, often with conspicuous clamps, skeletal hyphae dominating in the trama and context, unbranched or only very rarely branched, flexuous, thick-walled to solid, strongly dextrinoid, 3-6 µm

wide. Spores globose to truncate, 4.5-5.5 µm in diameter, thick-walled, smooth, pale yellowish and non-dextrinoid.

*Phanerochaete sordida* (Karst.) Erikss. and Ryv.

Specimens examined: THAILAND, Nakhon Ratchasima Province. Sakaerach, June 2008, collector P. Permpornsakul, Figure 4.1 (5)

Basidiocarp resupinate, attached loosely to the substrate, forming thin, 0.5 mm thick membranous patches several centimeters to decimeters in extent, surface smooth, velutinous and dull, whitish-cream to pale orchraceous, margin finely farinose-filamentous or in part distinctly bounded, without rhizomorphs. Consistency waxlike and soft, somewhat fissured when dry. Hyphal system monomitic, Subicular hyphae rather thick-walled, 2.5-6 5 µm across, sometimes incrusted with crystals, septa without clamps. Cystidia thin-walled, somewhat thicker-walled toward the base, smooth to strongly encrusted, 60-130 x 6-10 µm. Basidia slenderly clavate, 25-30 x 4.5-5 5 µm , with 4-sterigmata, without basal clamp. Spores narrowly ellipsoid, smooth, hyaline, sometimes with droplets, 5-7 x 2.5-3.5 µm.

*Phanerochaete velutina* (DC, Pers.) P. Karst.

Specimens examined: THAILAND, Nakhon Ratchasima Province. Sakaerach, June 2008, collector P. Permpornsakul, Figure 4.1 (2, 14)

Basidiocarp resupinate, attached loosely to the substrate, forming membranous patches several centimeters to decimeters in extent, up to 0.5 mm thick, surface smooth to slightly tuberculate, velutinous and dull, whitish-cream-colored, margin filamentous, sometimes with rhizomorphs. Consistency waxlike and soft, somewhat fissured when dry. Hyphal system monomitic, subicular hyphae thin-walled, 2.5-5 µm across, some sparsely incrusted with crystals, septa without clamps. Lamprocystidia thick-walled, upper half strongly incrusted, exserted beyond the hymenium, 50-100 x 10-15 µm . Basidia cylindrical-clavate, 28-38 x4-6 µm, without basal clamp, with 4 sterigmata. Spores ellipsoid, flattened on one side, smooth, hyaline, 5.5-6.5 x 2.5-3.5 µm.

### 4.3.1 Characterization of resupinate white rot isolates

Thirteen of 25 resupinate fungal isolates showed evidence of lignin-modifying enzyme production in substrate specific agar plate assays. The nucleotide internal transcribed spacer (ITS) region sequences of these white rot fungi were submitted to GenBank (Table 4.2). ITS sequence from each fungus was compared to the available nucleotide sequences in the GenBank database using a BLASTn search; organisms providing the highest nucleotide sequence similarity to each searched ITS sequence are provided in Table 4.2.

These 13 resupinate white rot fungi were further characterized by growing on different media and at different temperatures and correlated with lignocellulolytic enzyme productions (Table 4.3). Most of these isolates exhibited similar growth raets at 30˚C and 37˚C**.** Eight isolates of the thirteen grew faster at 37˚C than 30˚C. Only 3 isolates were capable of growth at 43˚C including the *P. chrysosporium*. Resupinate cultures on 2% of MEA produce a thinner mycelium, but thicker mycelium on 2% OMA compared to the mycelium on 2% of PDA. Thirteen isolates were positive for MnP activity, fourteen isolates were positive for Lac activity and eight isolates were positive for LiP activity. The cellulase activity was detected from most of them, except *Phanerochaete* sp. Cut3, *Irpex lacteus* Kan10, *Bjerkander adudta* Nan6 and *P. sordida* Sk7 which indicated a selective delignification capability.

All of Lignin peroxidase-producing isolaes were further investigated for production of lignocellulolytic enzymes in liquid media and assessed for an ability to decolorized structurally different synthetic dyes (Table 4.4). Only *P. sordida* Sk7 and *P. chrysosporium* could decolorize all eight synthetic dyes.

**Table 4.2** Isolation and identification of resupinate white rot fungi from Thailand

| Fungal strain name | Collection sites (Provinces) | Collection number | ITS accession no. | Maximum nucleotide sequence similarity (%) |
|---|---|---|---|---|
| *Bjerkandera adusta* Nan6 | Nan | PBU0047 | KC570339 | *B. adusta (95%)* |
| *Ceriporia cystidiata* Roi2 | Roi Et | PBU0048 | KC570340 | *Ceriporia lacerate* (98%) |
| *Irpex lacteus* Kan10 | Kanchanaburi | PBU0049 | KC570336 | *I. lacteus (99%)* |
| *Junghuhnia crustacea* Kan4 | Kanchanaburi | PBU0050 | KC570332 | *Basidiomycete sp. (93%)* |
| *Macrohyporia dictyopora* Kan1 | Kanchanaburi | PBU0051 | KC570338 | *Basidiomycete sp. (90%)* |
| *Macrohyporia* sp. Kan2 | Kanchanaburi | PBU0052 | KC570341 | *Basidiomycete sp. (90%)* |
| *Perenniporia tephropora* Sk13 | Nakhon Ratchasima | PBU0053 | KC570342 | *P. tephropora (96%)* |
| *Perenniporia tephropora* Skb1 | Nakhon Ratchasima | PBU0054 | KC570336 | *P. tephropora (96%)* |
| *Perenniporia sp.*Kao2 | Cholburi | PBU0055 | KC570330 | *P. corticola(97%)* |
| *Phanerochaete sordida* Sk7 | Nakhon Ratchasima | PBU0057 | KC570340 | *P. sordida (99%)* |
| *Phanerochaete velutina* Kao31 | Cholburi | PBU0058 | KC570339 | *P. chrysosporium (99%)* |
| *Phanerochaete* sp. Cut3 | Bangkok | PBU0056 | KC570337 | *P. sordida (99%)* |
| *Phanerochaete* sp. Kan7 | Kanchanaburi | PBU0059 | KC570333 | *P. chrysosporium (99%)* |

**Table 4.3** Characterization of resupinate white rot fungi from Thailand

| Scientific name | Growth[a] | | | Colony morphology[b] | | | Enzyme productivity[c] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 30◦C | 37◦C | 43◦C | PDA | MEA | OMA | LiP | MnP | Lac | Xyl | Cel |
| *B. adusta* Nan6 | 7D | 7D | - | White/A | A | SF | 5D<br>+ | 5D<br>++ | 4D<br>+ | + | - |
| *C. cystidiata Roi2* | 9D | 10D | - | White/C/SF | C/SF | C/F | - | 8D<br>+ | 2D<br>++ | + | ++ |
| *I. lacteus* Kan10 | 6D | 5D | + | Yellow/F | F | VF | 3D<br>++ | 5D<br>++ | 7D<br>+ | ++ | - |
| *J. nitida* Kan4 | 9D | 10D | - | White/SF | SF | F | 3D<br>++ | 7D<br>+ | 4D<br>++ | ++ | + |
| *M. dictyophora* Kan1 | 4D | 4D | - | White/F | F | VF | 4D<br>++ | 7D<br>+ | 5D<br>+ | ++ | ++ |
| *Macrohypoia* sp. Kan2 | 6D | 6D | - | White/A/C | SF | F | - | 7D<br>+ | 2D<br>++ | + | + |
| *P. alabamae* Hua1 | 6D | 7D | - | White/F | SF | F | - | 5D<br>+ | 2D<br>++ | - | + |
| *P. corticola* Skb1 | 6D | 6D | - | White/C/F | C/F | F | - | 7D<br>++ | 1D<br>+++ | - | + |
| *P. corticola* Sk13 | 6D | 6D | - | White/C/F | C/F | F | - | 7D<br>++ | 1D<br>+++ | - | + |
| *P. medulla-panis* Kao2 | 6D | 6D | - | White/F | F | F | - | 8D<br>+ | 1D<br>+++ | - | + |
| *P. chrysosporium* * | 4D | 3D | + | White/S | A/S | SF/S | 3D<br>++ | 7D<br>+ | 7D<br>+ | - | - |
| *P. velutina* Kan7 | 5D | 4D | - | White/S | A /S | SF/ S | 4D<br>++ | - | 5D<br>+ | + | + |
| *P. velutina* Kao31 | 4D | 3D | - | White/S | A/S | SF/S | 4D<br>++ | 7D<br>+ | - | + | ++ |
| *P. sordida* Sk7 | 4D | 3D | + | White/A | A | SF/S | 3D<br>+++ | - | 5D<br>+ | + | - |
| *Phanerochaete sp.* Cut3 | 4D | 3D | + | White/A | A | SF | 3D<br>++ | 7D<br>+ | 5D<br>+ | - | - |

[a] Average day to cover the plate of mycelium (D), Present of growth (+), Absent of growth (-)

[b] A = appressed mycelia; F = fluffy mycelia; S = spore; C = compact mycelia; SF = slightly fluffy mycelia; VF = very fluffy mycelia

[c] Average initial days to record enzyme activity (D), Intensity and/or diameter ratio of activity zone and mycelia zone (+), Absent of activity (-)

* Reference fungus, *Phanerochaete chsosporium* ATCC2725

**Table 4.4** Enzyme profiles of lignin-peroxidase producing fungi and they ability to attack synthetic dyes

| Fungal isolates | Lignocellulolytic enzymes activities (U/ ml)[a] | | | | | Decolorization of synthetic dyes[b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LiP | Lac | MnP | Xyl | Cel | PR | RB5 | RB | As | Co | Con | Met | Bro |
| *B. adusta* Nan6 | 0.009 | 0.030 | 0.317 | 0.006 | ND | + | + | + | + | + | + | + | - |
| *I. lacteus* Kan 10 | 0.325 | 0.301 | 0.020 | 0.008 | ND | + | - | + | + | + | - | + | - |
| *J. nitida* Kan 4 | 0.013 | 0.232 | 0.110 | 0.010 | 0.069 | + | - | + | - | + | - | - | - |
| *M. dictyoporia* Kan 1 | 0.257 | 0.128 | 0.006 | 0.011 | 0.105 | + | - | + | - | + | - | + | - |
| *P. chrysosporium* * | 0.170 | ND | 0.115 | ND | 0.005 | + | + | + | + | + | + | + | + |
| *P. sordida* Sk 7 | 0.415 | 0.119 | ND | 0.013 | ND | + | + | + | + | + | + | + | + |
| *P. velutina* Kan 7 | 0.012 | 0.210 | ND | 0.018 | 0.012 | + | - | + | + | + | - | - | - |
| *Phanerochaete* sp. Cut 3 | 0.017 | 0.122 | 0.013 | ND | ND | + | - | + | + | + | + | + | - |
| *Phanerochaete* sp. Kao31 | 0.032 | ND | 0.011 | 0.006 | 0.168 | + | - | + | + | + | - | - | - |

[a]Lgnin peroxidase in relation to substrates, LiP (Lignin peroxidase), Lac (Laccase), MnP (Manganese peroxidase), Xyl (Xylanase), Cel (Cellulase)

[b]The structurally different synthetic dyes: Pro (Procion red), RB5 (Reactive black 5), RRBBR (Remalzol brilliant blueR), Ast (Astrazon red), Coom (Coomassie blue), Cong (Congo red), Meth (Methylene blue), Brom (Bromothymol blue), ND (not detectable)

*Comparative level to *Phanerochaete chrysosporium* ATCC2725, reference fungus

### 4.4 Treatment of reactive black 5 dye

Reactive black 5 (RB5) is an azo dye representative of recalcitrant and toxic xenobiotic compounds, and thus was selected as the model substrate to evaluate fungal strains in decolorization assays. Among 8 lignin peroxidase-producing strains, including reference strain *P. chrysosporium* ATCC 24725. *P. sordida* Sk7 was able to decolorize RB5 at the fastest rate over a 3 day period (Table 4.5). Strain Sk7 decolorized 100 mg/l of RB5 in liquid media within 3 d of incubation without adsorption into mycelia. In contrast, *P. chrysosporium* decolorized RB5 to 97% in the same time period (Table 4.5). In the latter case, mycelial adsorption also played significant role in the decolorization. None of the other strains were able to reach a level above 80% decolorization during the same period. Because of *P. sordida* Sk 7 efficacy in decolorizing RB5, this strain was chosen for further characterization.

**Table 4.5** Decolorization of RB5 by tropical resupinate white rot fungi in liquid medium by day3

| Resupinate fungi | Collection number | RB5 decolorization (%) (Absorbance at 597 nm) |
|---|---|---|
| *Irpex lacteus* | PBU 0049 | 79 |
| *Junghuhnia crustacea* | PBU 0050 | 43 |
| *Macrohyporia dictyopora* | PBU 0051 | 61 |
| *Phanerochaete sordida* | PBU 0057 | 100 |
| *Phanerochaete* sp. | PBU 0056 | 63 |
| *Phanerochaete* sp. | PBU 0058 | 59 |
| *Phanerochaete* sp. | PBU 0059 | 48 |
| *Phanerochaete chrysosporium* | ATCC 24725 | 97 |

4.4.1 Effect of physicochemical culture conditions on RB5 decolorization by *P. sordida* Sk7

4.4.1.1 Effect of pH

The effect of *P. sordida* Sk 7 at three different pH values (6, 7 or 8) on the ability to decolorize RB5 showed that the initial rate of decolorization by *P. sordida* Sk 7 and *P. chrysosporium* were pH dependent. Under pH 6 and 7, the decolorization by *P. chrysosporium* reached a maximum at 96 % by day 4, but at the same day only 93 % decolorization was achieved at pH 8 (Figure 4.2b). In contrast with *P. sordida* Sk 7, although the initial rate of the decolorization was slightly delayed at pH 8, 100 % decolorization occurred by day 3 at all three pH values (pH 6-8) (Figure 4.2a).

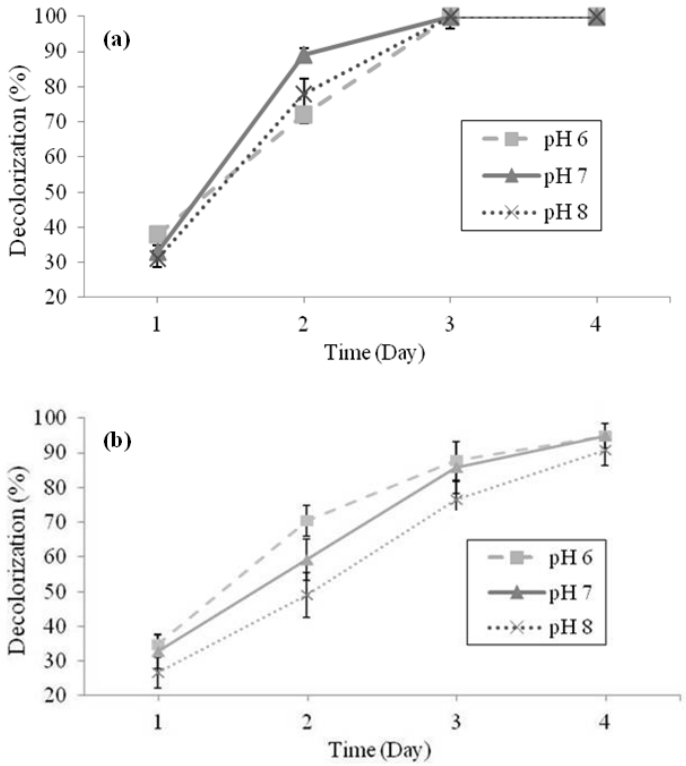



**Figure 4.2** Effect of initial pH on the decolorization of RB5 (100 mg $l^{-1}$) by (a) *P. sordida* SK7 and (b) *P. chrysosporium* ATCC 24725 at 30 $^{o}$C with agitation at 150 rpm. Data are mean ± SD of triple experiments.

4.4.1.2 Effect of Temperature

Incubation of fungal strains at three different temperatures (25, 30 or 35 °C) over a 4 day period affected RB5 decolorization by both *P. sordida* Sk7 and *P. chrysosporium* (Figure 4.3). Complete RB5 decolorization by *P. sordida* Sk7 was obtained by day 3 at both 30 and 35 °C, but was delayed until day 4 at 25 °C (Figure 4.3a), whereas decolorization by *P. chrysosporium* was slightly slower at high temperature (30 and 35 °C) (Figure 4.3b). (30 and 35 °C) (Figure 4.3b).

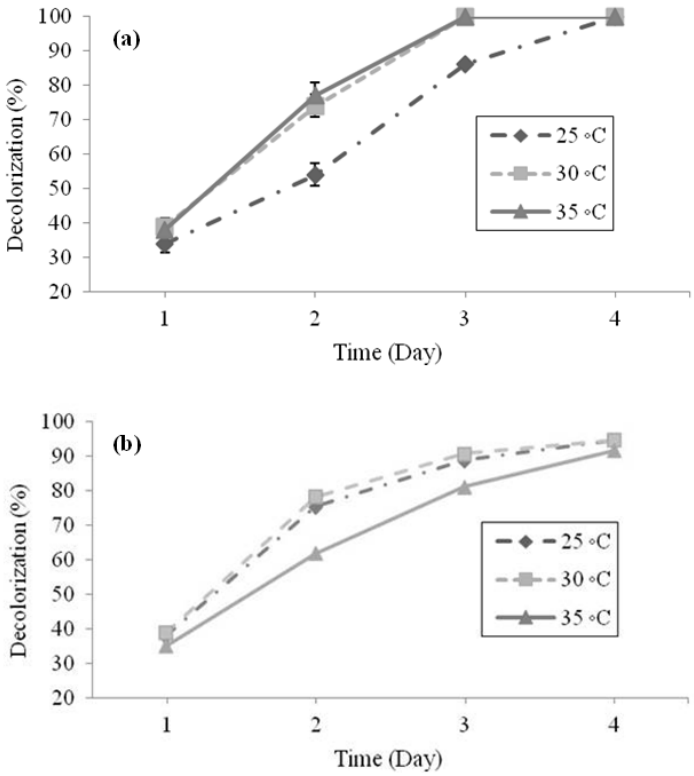



**Figure 4.3** Effect of temperature on the decolorization of RB5 (100 mg $l^{-1}$) by (a) *P. sordida* SK7 and (b) *P. chrysosporium* ATCC 24725 at pH 7 with agitation at 150 rpm. Data are mean ± SD of triple experiments.

4.4.1.3 Effect of agitation

Agitation (0 or 150 rpm) affected RB5 decolorization over a 4 d period, both *P. sordida* Sk 7 (100 %) and *P. chrysosporium* (97 %) were effective in still and shake culture (Figure 4.4). However, decolorization rate appeared to be greater in *P. chrsosporium* cultures incubated under static conditions compared with agitation conditions. Regardless, maximum decolorization (97 %) was achieved under both conditions by day 4(Figure 4.4b). In contrast, *P. sordida* Sk 7 decolorized RB5 slightly faster with agitation, reaching 100% decoloration by 3 d, compared with incubation under static conditions, which reached 100 % decoloration at day 4 (Figure 4.4a).

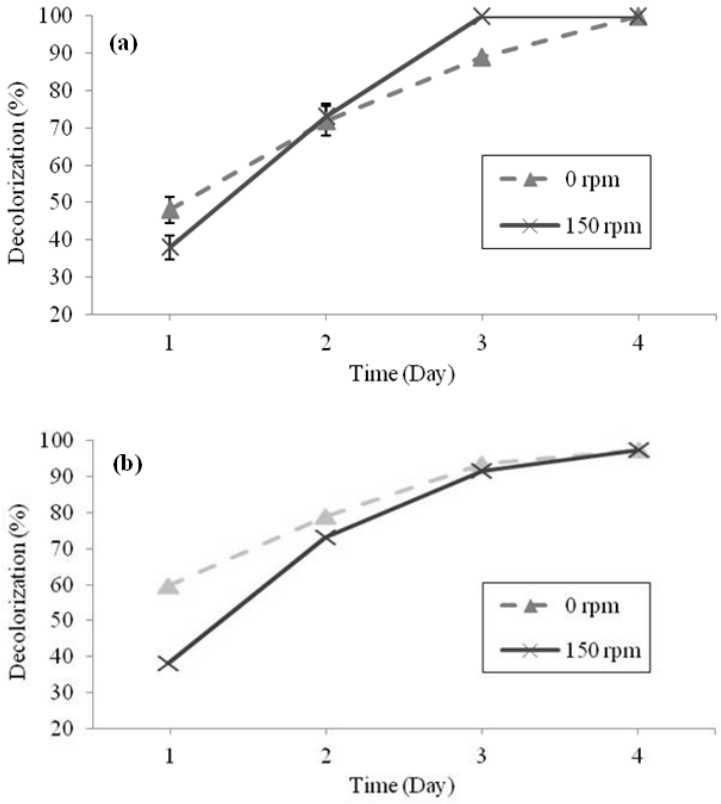



**Figure 4.4** Effect of agitation on the decolorization of RB5 (100 mg $l^{-1}$) by (a) *P. sordida* SK7 and (b) *P. chrysosporium* ATCC 24725 at 30 $^{o}$C and pH 7. Data are mean ± SD of triple experiments.

4.4.1.4 Effect of RB5 concentration

The effect of RB5 concentration was investigated using practical concentrations (100 and 200 $mgl^{-1}$). RB5 concentration clearly had an effect on the ability of *P. sordida* Sk7 to decolorized RB5. A much slower rate of RB5 decolorization was observed with Sk7 at the higher concentration of 200 $mgl^{-1}$ compared with 100 mg/l (Figure 4.5a). In contrast, RB5 concentration had much less to no effect on the ability of *P. chrysosporium* to decolorize the dye (Figure 4.5b). However, both yeilded the maximum decolorization of 200 $mgl^{-1}$ RB5 at the same day (day 4).

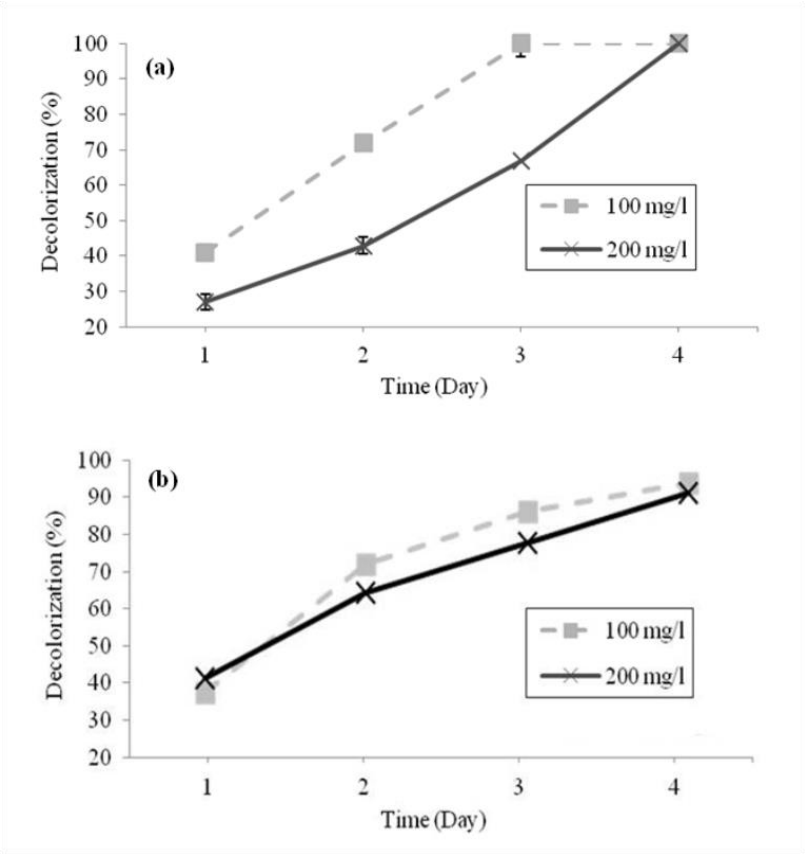



**Figure 4.5** Effect of RB5 concentration on the decolorization of RB5 by (a) *P. sordida* Sk7 and (b) *P. chrysosporium* ATCC 24725 at 30 °C, pH 7 with agitation at 150 rpm. Data are mean ± SD of triple experiments.

4.4.3 lignin-modifying enzymes levels during decolorization

Activities of laccase, LiP and MnP were evaluated daily over a 4 d period in culture fluids of *P. sordida* Sk7 and *P. chrsosporium* recovered from the RB5 decolorization assay. All three enzymes were produced during the decolorization by *P. chrysosporium*, whereas only laccase and LiP were found from the *P. sordida* Sk7 culture (Table 4.6). Over the 4 day incubation period of the decolorization assay, laccase activity increased gradually by *P. sordida* Sk 7. Similarly, gradual increases in both MnP and laccase activities were seen with the culture of *P. chrysosporium*. In contrast, LiP activity levels of both fungi were detected only in the early period of decolorization, with maximum levels reached by day 2 (Table 4.6).

**Table 4.6.** Lignin-modifying enzyme production of *P. sordida* Sk 7 and the reference strain, *P. chrysosporium* ATCC 24725 during the decolorization.

| | Enzyme activity (U $ml^{-1}$) | Day | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| *P. sordida* Sk 7 | Laccase | 0.34±0.02 | 0.36±0.02 | 0.39±0.03 | 0.44±0.03 |
| | LiP | 0.33±0.05 | 0.12±0.05 | 0.11±0.02 | 0.07±0.02 |
| | MnP | ND | ND | ND | ND |
| *P. chrysosporium* | Laccase | 0.10±0.01 | 0.14±0.02 | 0.17±0.02 | 0.21±0.02 |
| | LiP | 0.28±0.05 | 0.18±0.05 | 0.15±0.03 | 0.11±0.03 |
| | MnP | 0.21±0.03 | 0.27±0.04 | 0.29±0.04 | 0.31±0.03 |



4.4.4 Reactive black 5 dye degradation

Azo dye RB5 is blue in color and has a maximum absorbance observed at 597 nm (Figure 4.6). To verify that RB5 decolorization by *P. sordida* Sk7 coincides with degradation of the dye, fungal culture fluids were analyzed using both UV-visible light spectrophotometry and HPLC. The UV-visible light spectra revealed that a substantial absorption peak observed at 597 nm on day 0 in *P. sordida* Sk7 culture fluid samples decreased in size over time and became undetectable after 3 days (Fig. 4.6). Complete disappearance of the major peak of RB5 by day 3 of incubation suggested that *P. sordida* Sk7 has broken the azo bonds of the dye. HPLC analysis of filtrates from the control RB5 (Figure 4.7a) and cultures of *P. sordida* Sk7 (Fig. 4.7b) showed the disappearance of the major RB5 peaks and the appearance of a broad range of different peaks with different retention times. These peaks indicated the consistent of the decolorization of RB5 and the formation of degradative compounds.

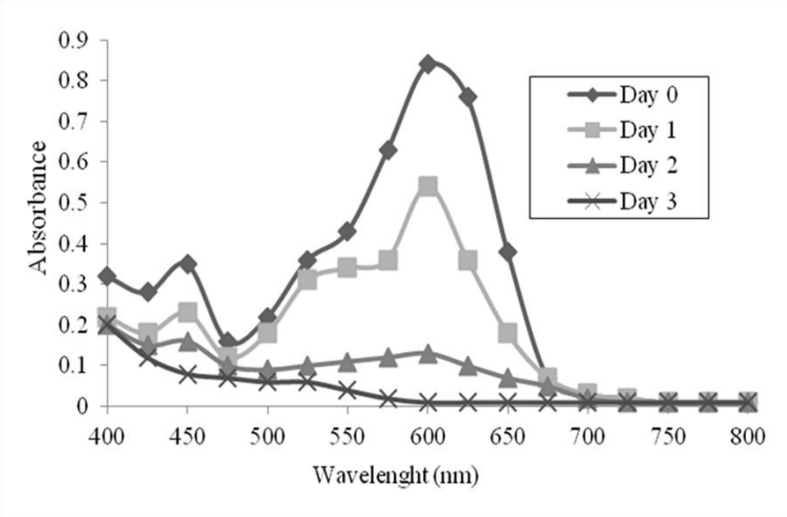



**Figure 4.6** The UV-vis spectrum of culture fluid containing RB5 after treatment with *P. sordida* Sk7 comparison from 0, 1, 2 and 3 days of the decolorization periods at pH 7 and 35 °C. Spectra are representative of two independent repeated experiments.

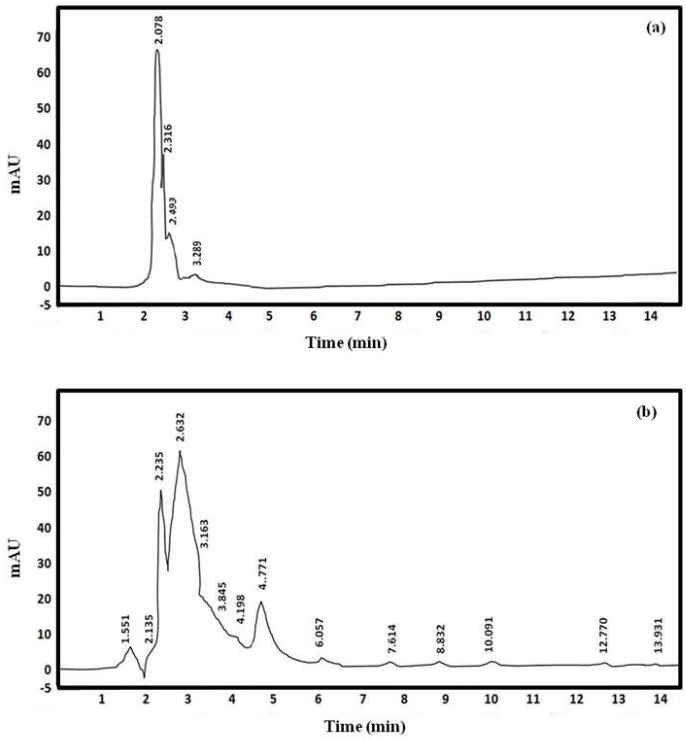



**Figure 4.7** HPLC profile of (a) RB5 (200 mg $l^{-1}$) and (b) the degraded RB5 after complete decolorization by *P. sordida* Sk 7 (RB5 at 200 mg $l^{-1}$ treated for 4 days at 30 $^{o}$C, pH 7 with agitation at 150 rpm). Profiles shown are representative of those seen from two independent experiments.

4.4.5 Phytotoxicity of Reactive black 5 degradation products

The toxicity of RB5 decolorized culture filtrates by *P. sordida* Sk7 was evaluated using seed germination phytotoxicity tests. Seeds of test plants were soaked decolorized dye metabolites were ranging from 90-100 % and not significant difference with the control (seeds soaked with water; 100 %), whereas they were significantly lower in the seeds exposed to original RB5 dye (40-80 %) (Table 4.7). Likewise for seedling growth, in terms of the shoot and root length, the seeds soaked with the exhibited no growth inhibition effect of the three test plants in contrast to the marked inhibition (Table 4.7) of seeds treated directly with the dye. An exception was red kidney bean shoot length that was not significantly different from the control. The toxicity towards plants of RB5 was reduced after treatment with *P. sordida* Sk7. These results indicated that decolorized filtrates of Sk7 cultures were less toxic than culture filtrates containing RB5.

**Table 4.7**. Phytotoxicity test of RB5 (200 mg $l^{-1}$) and its degradation products on *Sorghum bicolor* L., *Zea mays* L. and *Phaseolus vulgaris* L.

| Parameters | *Zea mays* L. | | | *Phaseolus vulgaris* L. | | | *Sorghum bicolor* L. | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | Untreated* | Treated** | Control | Untreated* | Treated** | Control | Untreated* | Treated** |
| Germination (%) | $100^{a}$ | $65^{b}$ | $90^{a}$ | $100^{a}$ | $80^{b}$ | $100^{a}$ | $100^{a}$ | $40^{b}$ | $90^{a}$ |
| Shoot length (cm) | $1.3\pm0.4^{a}$ | $0.7\pm0.1^{b}$ | $1.5\pm0.2^{a}$ | $1.4\pm0.3^{a}$ | $1.2\pm0.4^{a}$ | $1.3\pm0.4^{a}$ | $0.5\pm0.2^{a}$ | $0.1\pm0.2^{b}$ | $0.7\pm0.4^{a}$ |
| Root length (cm) | $4.3\pm0.6^{a}$ | $2.9\pm0.4^{b}$ | $4.6\pm0.6^{a}$ | $3.5\pm0.3^{a}$ | $2.5\pm0.6^{b}$ | $3.9\pm0.4^{a}$ | $2.0\pm0.4^{a}$ | $0.7\pm0.4^{b}$ | $2.4\pm0.6^{a}$ |



* Seeds soaked with RB5 (200 mg $l^{-1}$).

** Seeds soaked with RB5 treated by P. sordida SK7 (RB5 at 200 mg l-1 at 30 $^{o}$C with 150 rpm and pH 7 for 4 days to complete decolorize.

Values are the mean ± SD of five replicate determinations, Values followed by the different letter in the same row of each plant species are significantly different from the control (Seed soaked with water) at $p < 0.05$ by one-way analysis of variance (ANOVA) with Dunnett's test

### 4.5 Purification of lignin peroxidase from *P. sordida* Sk7

Crude enzyme was prepared from 1 liter of production medium. The crude extract from the culture filtrate yielded 350 U of lignin peroxidase activity (Table 4.8). Crude enzyme activity was condensed 10-fold by ultrafiltration (30 kDa molecular weight cut off, which yielded 100 ml with 292 U of lignin peroxidase activity (Table 4.8). The crude enzyme was recovered by ammonium sulphate precipitation by 4 stepwise increases from 45-85 % and showed 3.3 fold increase in activity and unknown contaminated was removed at this step. The protein remained 12 mg with 148 U of lignin peroxidase activity (Table 4.8). The enzyme solution obtained from ammonium sulfate precipitation was applied to DEAE anion exchange column (Figure 4.8). Lignin peroxidase activity was found only in fractions 24-28. These fractions were combined and concentrated by ultrafiltration. The purity of the enzyme was monitored by SDS-PAGE (Figure 4.9) and the enzyme solution from DEAE column showed a single band.

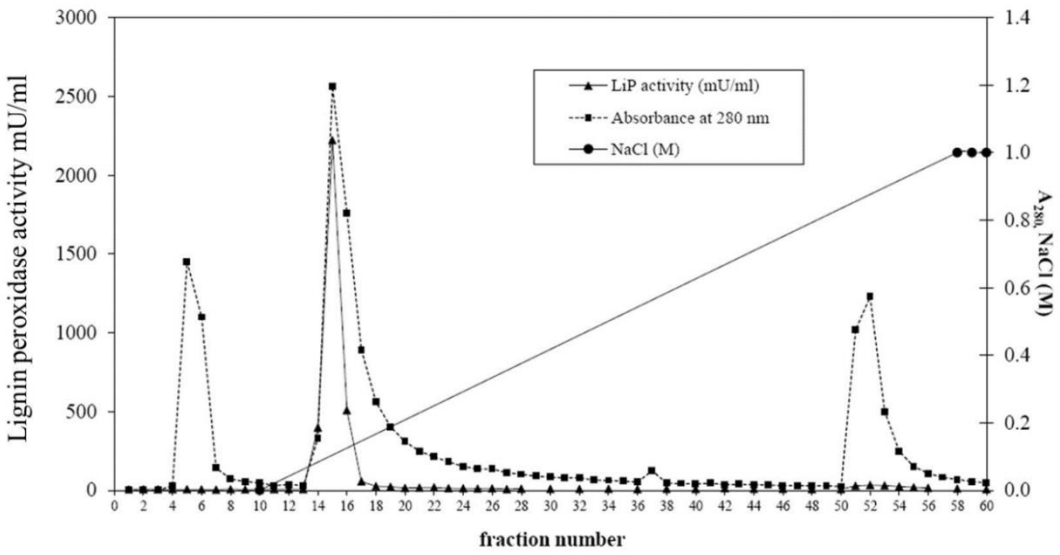



**Figure 4.9** An elution profile of lignin peroxidase from DEAE Sephadex G-100 column.

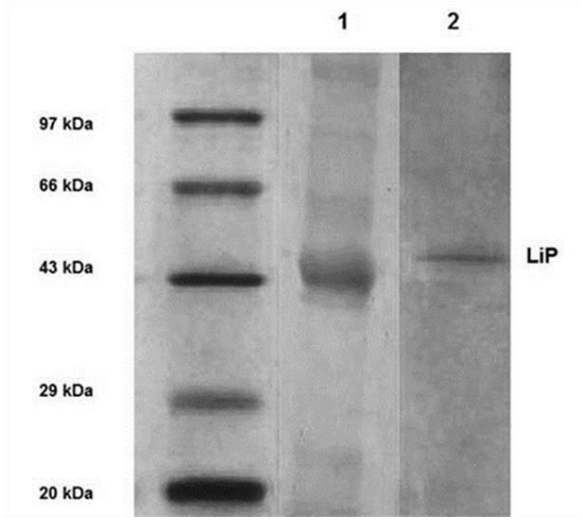



**Figure 4.8.** The SDS-PAGE analysis of lignin peroxidase from *P. sordida* Sk7. Lane 1: showed the enzyme solution obtained from ammonium precipitation 45-85% saturation and Lane 2: lignin peroxidase from DEAE column showed a single band with apparent molecular weight of ~45 kDa for pool 24-28 fraction of DEAE column.

**Table 4.9** Purification of lignin peroxidase from *P. sordida* Sk7

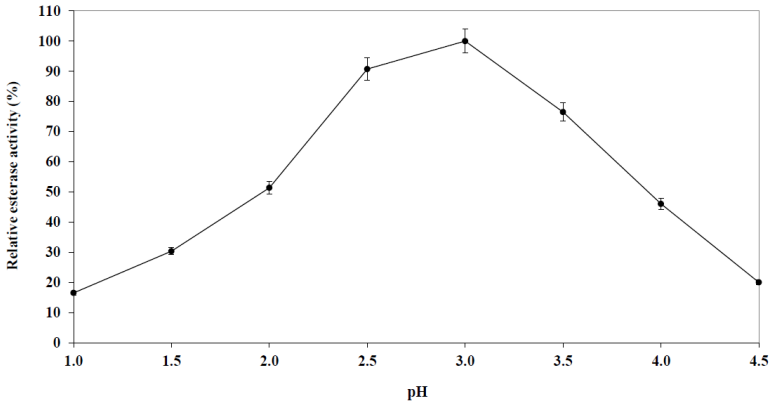
| Purification steps | Total Volume (ml) | Total Activity (U) | Activity (U/ml) | Total protein (mg) | Specific activity (U/mg) | Purification fold | % Recovery |
|---|---|---|---|---|---|---|---|
| Crude enzyme | 875 | 350 | 0.4 | 122 | 2.86 | 1 | 100 |
| Ultrafiltration (30 kD cut off) | 100 | 292 | 2.92 | 31 | 9.42 | 3.29 | 83.42 |
| Ammonium sulfate (45-85 %) | 30 | 148 | 4.93 | 12 | 12.33 | 4.31 | 42.29 |
| Anion exchange (DEAE Sephadex) | 20 | 74 | 3.6 | 5 | 22.87 | 8.00 | 21.14 |

## 4.6 Characterization of purified lignin peroxidase from *P. sordida* Sk7

### 4.6.1 Effect of pH on lignin peroxidase activity and stability

The optimum pH of the lignin peroxidase was determined using pH 2.0-4.0 of glycine-HCL buffer, pH 4.0-6.0 of sodium tartrate buffer and pH 6.0-7.0 of sodium phosphate buffer. Optimum pH of the lignin peroxidase was pH 3.0 (Figure 4.10a). The pH stability of the enzyme was assessed by incubating the enzyme at pH ranging from 2.0-7.0 for 30 min at 4°C and residual activity was measured. The enzyme was stable at pH 3.0 – 6.0 (Figure 4.10b).

(a)




(b)

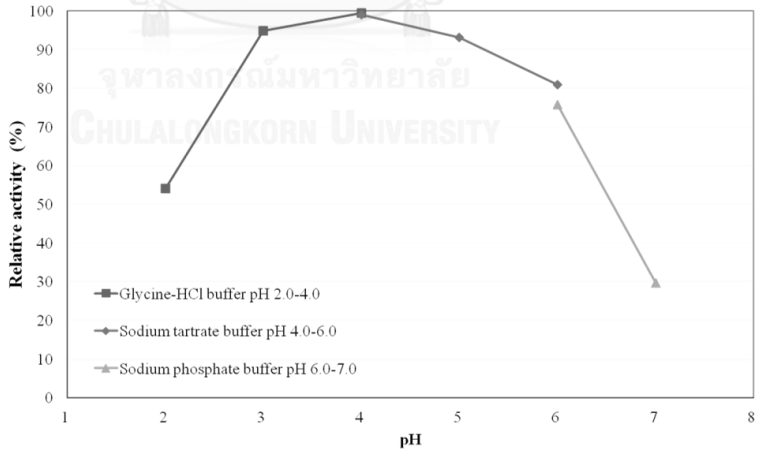
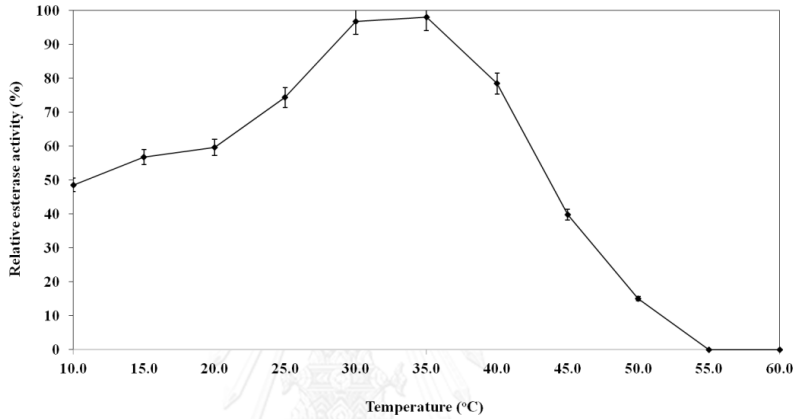



**Figure 4.10** Effect of pH on (a) lignin peroxidase acivity and(b)stability

4.6.3 Effect of temperature on lignin peroxidase activity and stabiliy

The optimum temperature of lignin peroxidasewas determined by performing the assay in the temperature range from 10-60 ˚C. The enzyme showed the greatest activity at 35 ˚C (Figure 4.11a). The effect of temperature on stability of the enzyme was determined by heating without substrate for 30 min at different temperatures. The enzyme activity remained stable when the enzyme was incubated at the temperature ranging from 10-40 ˚C for 60 min (Figure 4.11b)

(a)




(b)

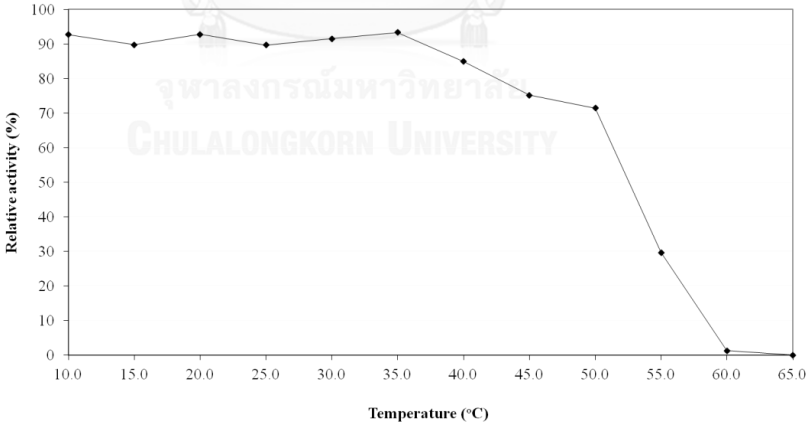





**Figure 4.11** Effect of temperature on (a) lignin peroxidase acivity and (b)stability

4.6.5 Effect of metal ions on lignin peroxidase activity

Effect of metal ions on lignin peroxidase activity including $Ca^{2+}$, $Cu^{2+}$, $Fe^{2+}$, $Hg^{2+}$, $Mg^{2+}$, $Mn^{2+}$ and $Ag^{+}$ was determined by adding the metal salts to the enzyme reaction mixture at different concentrations (Table 4.9.) The lignin peroxidase was stable to most salts with even over 80 % stability to Mercury. Manganese gave greater lost of activity at 77 %. Iron activated the enzyme at 175 %. The present of EDTA show the sensitivity toward loss of metal ion, nearly 100 % lost of activity with the present of 10 mM EDTA.

**Table 4.9** Effect of metal ions and EDTA on lignin peroxidae activity

| Substances | Relative activity (%) | | |
|---|---|---|---|
| | Ion Concentration (mM) | | |
| | 0.1 | 1.0 | 10 |
| Control | 100 | 100 | 100 |
| $CaCl_2.2H_2O$ | 98.17 | 95.03 | 94.18 |
| $CuSO_4.5H_2O$ | 105.00 | 107.05 | 135.00 |
| $FeSO_4.7H_2O$ | 172.00 | 177.00 | 175.00 |
| $HgCl_2$ | 89.50 | 88.64 | 81.16 |
| $MgSO_4.7H_2O$ | 98.74 | 96.23 | 94.41 |
| $MnSO_4.7H_2O$ | 91.78 | 77.00 | 67.72 |
| $ZnSO_4.7H_2O$ | 99.94 | 95.61 | 94.92 |
| EDTA | 85.15 | 42.00 | 0.32 |

4.6.6 Effect of substrate concentration

Effect of substrate concentration of lignin peroxidase was determined by the Michalis-Menten kinetic constants Km and Vmax from Michalis-Menten and Lineweaver–Burk analysis (Figure 4.12). Concentrations of veratryl alcohol ranging from 0.1–1 mM were used. From x-intercept value (-1.6667 = $-1/K_m$), $K_m$ was calculated to 60.02 mM. $V_{max}$ was determined from y-intercept value (0.5535 = $1/V_{max}$) and it was calculated to 1.81 μmoles/min.

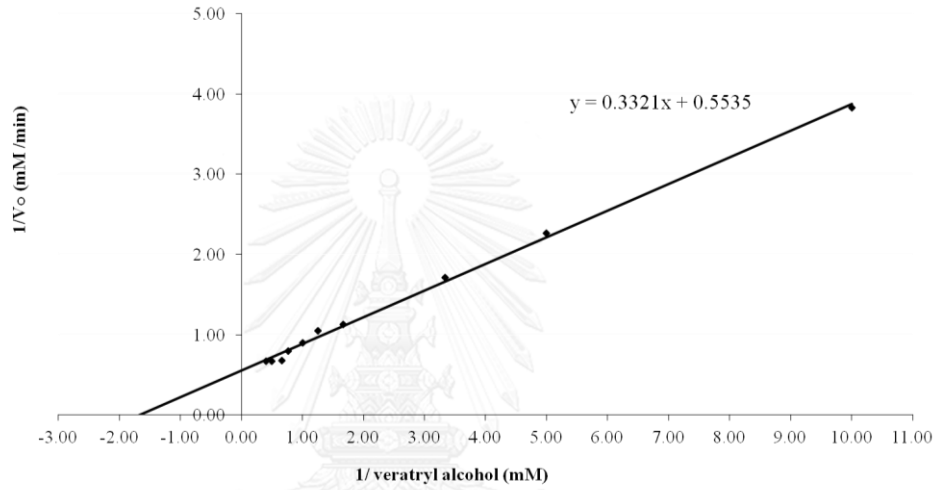



**Figure 4.12** Lineweaver-Burk analysis of lignin peroxidase of *P. sordida* Sk7 using veratryl alcohol as substrate

### 4.7 Sequencing of a lignin peroxidase cDNA gene

A full lenght lignin peroxidase cDNA gene was obtained using the RACE-PCR (rapid amplification of cDNA ends PCR) technique. It showed 92% similarity with the reported LiP H8 from *P. chrysosporium* (GenBank accession No. M74229), and 85% similarity with *P. sordida* ylp A (GenBank accession No. AB455006). Based on the sequences of the catalytic domain, primers were designed as

Forward primer: 5'-CCGCAGATGAACTTCTTCAC-3'

Reverse primer: 5'-GGAAGATGAACTGGAAGT-3'

The 5' and 3' regions were extended using the RACE-PCR technique (Ambion, First choice RACE kit, NJ). The 3'-region was cloned using the forward primer and the 3'-RACE primer, while the 5'-region was amplified using the 5'-RACE primer with another specific reverse primer (R2: 5'-GAGGAAGTCGGACTGGAGAC-3'). A final of 1172 bp fragment of the 5'-region and 978 bp fragment of the 3'-region were obtained, and the 5'- and 3'-flanking regions were assembled to synthesize the full-length cDNA gene (Appendix E). The primers for the RACE-PCR were presened in the full cDNA gene. The predicted ORF consisted of 361 amino acids from the 1,130 bp cDNA (Appendix E). When the nucleotide. The predicted amino acid sequences were analyzed through the BLAST program, the cDNA sequence and the deduced amino acid sequence of the ORF showed 92% identity and 98% similarity, respectively with the Lip isosyme H8 of *P. chrysosporium* (GenBank accession no. AAB00798).

# CHAPTER V

# DISCUSSION AND CONCLUSIONS

## 5.1 Isolation and identification of resupinate white rot fungi from Thailand

Resupinate fungi are an outstanding wood decomposer group (Kirk and Chang 1975). More than 200 resupinate fungal species were reported from an intensive survey at Nan province, Northern part of Thailand (Hjortstam and Ryvarden 1982, Choeyklin, Hattori et al. 2011) . In my study, 53 specimens were obtained from a further general survey in seven forested areas around Thailand (Figure 3.1; Table 4.1). They are widely represented Thailand as wood saprothrops. Buckley et al., 2007 notes that tropical fungi could tolerate to high temperature ranging from 22-35 ˚C as well as survive under high competitive environment of topical forest (Buckley, Palakit et al. 2007). Although, similar in gross appearance, the basidiocarps have distinctive diverse anatomical, physiological and molecular characteristics (Binder, Hibbett et al. 2005). However, the study of resupinae fungi has been limited in comparison to their non-resupinate counterparts. One general reason could be in regard to the difficulty in their isolation. In this study, only 25 of 53 resupinate specimens were recovered (Table 4.1). Part of the difficulty could be due to a very thin and fragile basidiocarp along with the open spore-bearing surface that is readily contaminated by fast growing competing fungi (Larsson 2007, Wu, Nilsson et al. 2010) . There are relatively few taxonomic experts of this group (Eriksson and Ryvarden 1975, Gilbertson and Ryvarden 1986, Wu 1990). Better selective isolation approach should be considered in order to obtain more information of the resupinate fungi e.g. addition of alternate inhibitors and incubation in high temperature.



## 5.2 Characterization of resupinate white rot fungi from Thailand

Screening for lignin-modifying enzymes (LMEs) revealed that lignin peroxidase which rarely found in white rot fungi (Hatakka and Hammel 2011), is common in this resupinate collection (Table 4.3). Six isolates of the thirteen peroxidase-producing resupinate white rot fungi had optimum growth temperature at 30˚C but as seen in tropical strain (Table 4.3), they could grow at high temperature. Three isolates were thermotolerant being able to grow at 43˚C (table 4.3). Culturing these isolates on 1.5% of malt agar yeilded thin compact mycelia (Table 4.3). This status was optimal for the preparation of inocula for further production and enzymatic studies (Pointing 2001). Most white-rot fungi are able to degrade all the wood polymers. However, 4 isolates (*Phanerochaete* sp. Cut3, *Irpex lacteus* Kan10, *Bjerkandera adusta* Nan6 and *P. sordida* Sk7) preferentially produced lignin and hemicellulose-degrading enzymes, while leaving cellulose polymer almost intact (Table 4.3)

(Eriksson, Blanchette et al. 1990) (Kuhad, Singh et al. 1997). Selective lignin degraders include *Dichomitus squalens, Physisporinus rivulosus,* and *Ceriporiopsis subvermispora* (Hakala, Lundell et al. 2005, Fackler, Schwanninger et al. 2007). The model white rot fungus, *Phanerochaete crysosporium* (Martinez, Larrondo et al. 2004) is efficient in wood and lignocellulose decay but less selective for depolymerization of lignin over cellulose utilization (Hatakka and Uusi-Rauva 1983, Akhtar, Blanchette et al. 1997, Hakala, Maijala et al. 2004). Lignin peroxidase production by *P. sordida* Sk7 was almost 3-fold greater than *P. chrysosporium* (Table 4.4). This could be a useful properties.

5.3 Decolorization of synthetic dyes by lignin peroxidase-producing resupinate fungi.

The lignin peroxidase-producing white rot fungi were characterized for the biodecolorization toward structurally different synthetic dyes. The dye structures will affect the decolorization capability of the fungus (Ali, 2010). The dyes may require a higher redox potential of catalyst to trigger electron distribution (Knapp, Newby et al. 1995). *P. sordida* Sk7 and *P. chrysosporium* can decolorize a variety of dye structures including diazo, triazo, anthraquinone and triphenylmethane (Pavko 2011).

5.4 Treatment of RB5 by *P. sordida* Sk7

A choice of RB5 as a model synthetic dye was based on that it is most common azo dye used in the industry. It contains a sulfonate structure that is resistant to microbial attack. This may also retard microbial growth, for example by inhibiting nucleic acid synthesis (Asad, Amoozegar et al. 2007). The Thai tropical resupinate white rot fungi appeared to have a good potential for azo dye decolorization (and so potential in degradation) since this screening revealed 7 out of 13 could achieve effective decolorization of RB5 (from 100 mg/l) by up to 50 % removal of the color were achieved within 3 days (Table 4.5). Decolorization by several resupinate fungi including *Ceriporia cystediata, Junghuhnia crustacean, Macrohyporia dictyopora* and *Perenniporia tephropora* is apparently reported for the first time (Table 4.5).

The complete removal of the 100 mg/l RB5 in liquid media was achieved within 3 days of incubation by *P. sordida* Sk7 (Table 4.5) and this ability was slightly greater than the previous report *of P. chrysosporium* (93 % decolorization of 100 mg/l RB5 within 4 days). It is noted that this comparison is dependent on inoculum size and physiological state of the inoculum. The fungal decolorization mechanism could be by biodegradation and/or biosorption. Biodegradation breaks down the dye structure resulting in permanent dye removal, although it may yield toxic or inert compounds, whereas adsorption temporarily removes the dyes which could be released to the

environment following later mycelial degradation. Monitoring the dye within the mycelia failed to detect RB5 in *P. sordida* Sk7. after the complete decolorization. Adsorption played no significant role in the decolorization. A similar result was reported for the decolorization of textile dye wastewater by *Bjerkandera adusta* MUT 3060 (Anastasi, Parato et al. 2011).

Wastewater effluents from the azo dye residues is usually in the range of neutral to alkaline (Pavko 2011). *P. sordida* Sk7 efficiently decolorized RB5 in a basic environment (pH 6-8) (Figure 4.2). The pH tolerance of the fungus is a matter of concern since fungi typically prefer a weakly acidic condition for growth (Saratale, Saratale et al. 2009). In my studies, *P. sordida* Sk7 was exceptional since it could completely decolorize RB5 at a slightly acidic pH as well as neutral and slightly alkali conditions (Figure 4.2).

*P. sordida* SK7 gave maximal RB5 decolorization at 30-35 $^{o}$C (Figure 4.3). This temperature range is realistic for many dye effluents. The temperature clearly affects the decolorization activity of each fungus in regard growth of tropical fungi (Hadibarata, Adnan et al. 2013). The RB5 decolorization ability of *P. sordida* SK7 under agitated or static culture do not differ (Figure 4.4) which allows practical applicability of either conditions. Yet in contrast, agitation can either enhance azo dye decolorization efficacy (*Pleurotus eryngii* F032) or retard such efficacy of *P. chrysosporium* and *Irpex lacteus* (Bakshi, Gupta et al. 1999, Svobodová, Erbanová et al. 2006, Hadibarata, Adnan et al. 2013).

*P. sordida* SK7 completely decolorized RB5 at 100 and 200 mg/l (Figure 4.4) which is in a practical range of dye concentration in the textile industry effluent (Kaushik and Malik 2009) . The increased time for total decolorization of RB5 by *P. sordida* SK7 with the increased initial dye concentration (Figure 4.4) is expected, and generally reported in *P. chrysosporium, Pycnoporus eryngii* F032 and *Trichosporon beigelii* (Saratale, Saratale et al. 2009, Hadibarata, Adnan et al. 2013). However, as mentioned above, the average rate of RB5 degradation can increase at the higher RB5 concentration (1.2- to 1.50-fold)

The activity of LMEs (as laccase, LiP and MnP) produced by *P. sordida* SK7 during RB5 decolorization (RB5 at 100 mg/l, pH 7, 30 $^{o}$C with agitation at 150 rpm for 3 d) was investigated in order to provide information on the potential dye decolorization mechanisms utilized by this fungus. Only LiP and laccase, and none of MnP, activities were detected (Table 4.6). This contrasts with a temperate strain of *P. sordida* that produced mainly MnP and LiP (Rüttimann-Johnson, Cullen et al. 1994). During the RB5 decolorization, LiP activity could be detected only in the early period of decolorization, whereas laccase activity appeared to correlate with the decolorization (Table 4.6).

Both LiP and laccase have previously been linked to the decolorization ability of azo dyes by white rot fungi (Hadibarata, Adnan et al. 2013), but the decolorization of RB5 by *P. sordida* SK7 perhaps occurred mainly through the activity of laccase. However, such a correlation on its own does not establish the role for laccase, but for the potential enhancing involvement of LiP in providing the LiP-produced metabolites which further metabolized by laccase.

The maximum absorbance of RB5 is 597 nm (Figure 4.6). The conjugated ¶-system that links the two azo bonds when broken results in the loss of the dye color. This major peak of RB5 (597 nm) completely disappears following decolorization, indicating the dye structure had been broken (Gonzalez-Gutierrez and Escamilla-Silva 2009).

This notion was supported by the HPLC analysis, where the disappearance of the RB5 peaks and the formation of multiple new peaks were shown (Figure 4.7). Biodegradation of either RB5 by *P. chrysosporium* (Enayatizamir, Tabandeh et al. 2011) and reactive Levafix Blue by *Irpex lacteus* (Kalpana, Shim et al. 2011) were confirmed by HPLC analysis (Figure 4.7). An initial dye of 200 mg/l was used to ensure a sufficient amount of the less common degradation products for their detectable (Zhao and Hardin 2007).

The degradation of RB5 by *P. sordida* Sk7 could, however, lead to the formation of more toxic products and so dispersing of treated dye wastewater to an environment may have an impact on soil fertility, and by to extension agricultural productivity (Gonzalez-Gutierrez and Escamilla-Silva 2009) . Ecotoxicological tests using plants have been used as indicators to assess the toxic potential of various contaminants, and the efficacy of a remediation process (Cruz, Lopes et al. 2013). The assay was based on seed germination percentage and seedling growth, in terms of root and shoot length. It has been proposed that azo dyes and their degradation products cause oxidative stress resulting in the induction of reactive oxygen species-scavenging enzymes in the plants (Puvaneswari, Muthukrishnan et al. 2006). This could cause reduced seed germination and reduced elongation of the seedling shoot and root (Table 4.7). However, the degraded RB5 products from *P. sordida* Sk7 were less toxic compared to the original dye. The biodegradation of diazo dyes by *P. sordida* Sk7 resulted in ITS detoxification and generation of nontoxic products. Similar detoxification by fungi of the azo dye Reactive Red, towards *Sorghum bicolor, S. vulgare, Phaseolus mungo*, and *Triticuma estivum*, has been reported were markedly reduced after degradation by white rot fungi (Saratale, Saratale et al. 2009, Adnan, Hadibarata et al. 2015).

5.5 Purification of LiP (lignin peroxidase) from *P. sordida* Sk7

Enzyme purification based on size, charge, solubility and specific binding properties. Purification of LiP enzyme required several steps (Table 4.8). The key is to select the most appropriate techniques, optimize their performance and combined them in logical way to maximize the yield and minimize the number of steps required (Ersson, Rydén et al. 2011). Ammonium sulfate precipitation is precipitates the protein in part according to molecular size. Ion Exchange Chromatography can be an effective separation technique by using DEAE anion exchange column since only one major peak was found (Figure 4.9). However, the amount of lignin peroxidase isozymes appeared to be vary in each fungus including ten isozymes were found from *Phanerochaete chrysosporium* (Tien, Kirk et al. 1988), two isozymes were found from *Pleurotus sajor caju* (Yadav, Singh et al. 2009) and one isozyme was found from *Loweporus lividus* (Yadav, Yadav et al. 2009). The purification result gave a specific activity of 22.87 U/mg and a 21.14-fold purification step. However, almost 50 % of total lignin peroxidase activity was lost during the ammonium precipitation and ion exchange steps.

5.6 Characterization of purified LiP from *P. sordida* Sk7

*P. sordida*, together with *B. adusta, P. chrysosporium,* and *P. radiate* belong to the resupinate fungal group known to produce lignin peroxidase (LiP) (Niku-Paavola, Karhunen et al. 1990, Hatakka 1994, Lundell and Hatakka 1994). *P. chrysosporium* is well characterized fungus that secrete a suite of multiple lignin-modifying peroxidases (LiPs and MnPs) with concomitant laccase production (Johansson and Nyman 1993, Hatakka 1994). This study, proposes a new LiP-cDNA of *P. sordida* Sk7. This is based on gene sequence analysis (Appendix E.) Results in this isozyme has not yet cloned or characterized and it is a useful future goal. Addition of a high gene concentration (480 μM) of soluble $Mn^{2+}$ to the semi-solid wood cultures inhibited expression of the Pr-lip3 gene. LiP3 is the predominant isozyme in *P. radiata* (Niku-Paavola, Karhunen et al. 1990). It appeared that optimum lignin peroxidase activity was obtained at pH 3.0 with decrease in activity at pH 5. A distinctive feature was its low pH optima. Increase in pH resulted in the disruption of the hydrogen bond formed between the heme propionate and aspartic acid residue in the active site of the enzyme with resulting in inactivation (Edwards, Raag et al. 1993). The optimum for this LiP of *P. chrysosporium* is in the acidic pH range (3-5) (Tien and Kirk 1984). The purification step used in this study (Table 4.8) similar to a purified lignin peroxidase from *Loweporus lividus* MTCC1178. (Yadav, Yadav et al. 2009). SDS-PAGE revealed a molecular mass of *P. sordida* Sk7 at 45 kDa (Figure .8). The molecular mass of lignin peroxidase from white rot fungi are in the range of 37-50 kDa (Farrell, Murtagh et al. 1989, Hirai, Sugiura et al. 2005).

The LiP activity in relation to pH from *P. chrysosporium* (Farrell, Murtagh et al. 1989) suggested that though higher enzyme activities were expressed at low pH (3) as well as the range of pH stability from 3-6 were also similar to the previous report of *P. chrysosporium* (Tuisel, Sinclair et al. 1990). The enzyme was stable for 1 hour over a range of temperatures, 10˚C to 50˚C. After 50˚C, there was a gradual loss in activity. According to Michaelis–Menten and double reciprocal plots of the LiP from *P. sordida* Sk7 to veratryl alcohol (Figures 4. 12), $K_m$ values of the enzyme was 60 which is similar to $K_m$ values reported for the lignin peroxidase of *P. chrysosporium* (Tuisel, Sinclair et al. 1990). However, it was found that optimum temperature (30˚C) of the *P. sordida* Sk7 LiP was higher than *P. chrysosporium* (26˚C)(Tuisel, Sinclair et al. 1990) and *Pleurotus sajor-caju* (20˚C)(Yadav, Singh et al. 2009). EDTA, $Mn^{2+}$ and Hg were inhibitors, whereas $Fe^{2+}$ and $Cu^{2+}$ were activators of activity. EDTA was strongly inhibitory, mechanism of inhibition vary depending on the concentration (Chang and Bumpus 2001).

5.7 Full-length LiP cDNA from *P. sordida* Sk7

The full-length LiP cDNA of *P. sordida* Sk7 was obtained (see also Appendix E.). *P. sordida* Sk7 was grown in Mn-free Kirk medium in order to upregulate lignin peroxidase expression and suppress manganese peroxidase expression (Machii, Hirai et al. 2004). Thus, LiP activity was detected as the main lignin-modifying enzyme, Though MnP and laccase activities were scarcely detectable. The full-length LiP cDNA data for *P. sordida* Sk7 and the reference fungus, *P. chrysosporium* were obtained from the RACE-PCR technique. The LiP enzyme was predicted to encode a protein with highly similarities of the LiP isosyme H8 from *P. chrysosporium* RP-78 (Wariishi and Gold 1990). Future cloning and expression of this *P. sordida* LiP should reveal interesting detail comparison.

# CONCLUSIONS

This study revealed that Thailand has a variety of resupinate fungi. They are a valuable bioresource of tropical white rot fungi. Tropical isolates have several interesting unique characteristics of compared to the temperate strains, especially in regard to biotechnological potential, These include as a source of efficient LMEs, lignin peroxidase which appear rare in white rot fungi, thermotolerance. A variety of enzyme-producing patterns, high growth rate. Good yields of enzyme production, unique enzymatic systems such as selective delignification, effective biodegradation toward a range of recalcitrant xenobiotic compounds.

The newly isolated tropical resupinate white rot fungus, *Phanerochaete sordida* Sk7 can be regarded as an interesting candidate in biotechnology. It could be applicable in pulp and paper bleaching and also in bioremediation. Its high temperature tolerance at 43 ˚C could be further useful selective property. Decolorization of waste dyes material is via actual degradation and not simply due to, biosorption on the mycelium. A wide range of pH, temperature and dye concentration and also completely degradation toxic dye products appear nontoxic to the plants.

Lignin peroxidase from *P. sordida* Sk 7 is stable in acidic pH 3-6 and also over 10-50 ˚C. A low value of Km and a high $V_{max}$ of the lignin peroxidase for veratryl alcohol suggests high substrate affinity and catalytic efficiency. The Initial performance of *P. sordida* Sk7 LiP-related enzyme system in dye biodegradation appears to indicate that it has greater activity than lignin peroxidase of *P. chrysosporium*. The full-length cDNA transcript was similar to LiP isozyme H8 of from *P. chrysosporium*. These combined data suggest that *P. sordida* Sk7 can be regarded as good candidate of white rot fungi in bioremediation.

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# APPENDICES

## Appendix A

## Culture media

Each recipe contains 1 litre of medium:

1. Selctive medium agar

| | | |
|---|---|---|
| Malt extract | 30 | g |
| Benomyl | 0.01 | g |
| Rose Bengal | 0.01 | g |
| Chloramphenicol | 0.1 | g |
| Agar | 15 | g |

2. Potato Dextrose Agar (PDA)

| | | |
|---|---|---|
| Potato | 200 | g |
| Glucose | 20 | g |
| Agar | 15 | g |

3. Malt Extract Agar (MEA)

| | | |
|---|---|---|
| Malt extract | 20 | g |
| Glucose | 10 | g |
| Peptone | 0.5 | g |
| Agar | 15 | g |



4. Oatmeal Agar (OMA)

| | | |
|---|---|---|
| Oatmeal | 20 | g |
| Agar | 15 | g |

5. Malt Extract Broth (MEB), pH 5 (Diwaniyan et al., 2010)

| | | |
|---|---|---|
| Malt extract | 20 | g |
| $KH_2PO_4$ | 0.5 | g |
| $CaCl_2.2H_2O$ | 0.5 | g |
| $MgSO_4.7H_2O$ | 0.5 | g |

6. Basal medium for cellulase and xylanase pH 5 (Tsiklauri et al., 1999)

| | | |
|---|---|---|
| Ammonium tartrate ($C_4H_{12}N_2O_6$) | 5.0 | g |
| Glucose | 10.0 | g |
| $KH_2PO_4$ | 0.2 | g |
| $NH_4NO_3$ | 0.6 | g |
| $Na_2HPO_4$ | 0.5 | g |
| Yeast Extract | 1.0 | g |
| $FeSO_4.7H_2O$ | 0.06 | g |
| $CaCl_2.2H_2O$ | 1.0 | mg |
| $MgSO_4.7H_2O$ | 1.0 | mg |
| $ZnSO_4.7H_2O$ | 1.0 | mg |

7. Lignin modifying enzyme basal medium (LBM) pH 5

| | | |
|---|---|---|
| $KH_2PO_4$ | 2.0 | g |
| $MgSO_4.7H_2O$ | 0.5 | g |
| $CaCl_2.2H_2O$ | 0.1 | g |
| $C_4H_{12}N_2O_6$ | 0.5 | g |
| Glucose | 0.8 | g |
| Nitrilotriacetate | 0.15 | g |
| 2,2-dimethylsuccinic acid | 1.46 | g |

Thaimine-HCl 1 mg (add after autoclaving from a filter-sterilized stock)

8. Trace element solution

| | | |
|---|---|---|
| NaCl | 10 | mg |
| $MnSO_4.5H2O$ | 5.0 | mg |
| $CoCl_2.6H2O$ | 1.0 | mg |
| $FeSO_4.7H_2O$ | 1.0 | mg |
| $ZnSO_4.7H_2O$ | 1.0 | mg |
| AlK $(SO_4)_2$ | 0.1 | mg |
| $CuSO_4.5H_2O$ | 0.1 | mg |
| $H_3BO_3$ | 0.1 | mg |
| $NaMoO_{4.}2H_2O$ | 0.1 | mg |

9. Mn-free Kirk's basal medium, pH 4.5 (Tien and Kirk 1988)

| | | |
|---|---|---|
| Ammonium Tartarate | 0.22 | g |
| $KH_2PO4$ | 0.21 | g |
| $MgSO_4.7H_2O$ | 0.05 | g |
| $CaCl_2$ | 0.01 | g |
| Thiamine | 0.001 | g |
| Tween 80 (10%) | 10 | ml |
| 100 mM veratryl alcohol | 10 | ml |
| Trace element Solution | 10 | ml |

## Appendix B

## Reagent

1. 100 mM Tartrate buffer (pH 3) 500 ml

| | | |
|---|---|---|
| Tartaric acid | 3.84 | g |
| Sodium tartarate | 5.6 | g |

Make the final volume to 500 mL in distilled water

2. 4 mM Veratryl alcohol 500 ml

| | | |
|---|---|---|
| 3,4-dimethoxybenzyl alcohol | 0.44 | g |

3. 0.2 M $H_2O_2$ 500 ml

Pouring 1 ml of $H_2O_2$ in 500 ml distilled water, followed by thorough mixing

4. 1M Tris-HCl buffer 1 L

| | | |
|---|---|---|
| Tris base | 121.1 | g |
| $ddH_2O$ | 800.0 | ml |

Dissolve Tris in $ddH_2O$ and adjust pH with conc. HCl to pH 8.0

Bring to 1 L with distilled water (Do not autoclave)

5. 3M NaCl 1 L

| | | |
|---|---|---|
| NaCl | 125.3 | g |

Bring to 1 L ml with distilled water

6. 3 M Sodium acetate buffer, pH 5.2 100 ml

| | | |
|---|---|---|
| Sodium acetate | 408.3 | g |
| dH2O | 800 | ml |

Adjust to pH 5.2 with glacial acetic acid

| | | | |
|---|---|---|---|
| 7. 10% SDS | 1 L | | |
| | Electrophoresis grade SDS | 100.0 | g |
| | Add to 900 dd$H_2O$ when SDS has dissolved | | |
| 8. 0.5 M EDTA, pH 8.0 | 1 L | | |
| | $Na_2$ EDTA. $2H_2O$ | 186.1 | g |
| | Bring pH to 8.0 with NaOH pellets (About 20 g) | | |
| | The EDTA will not dissolve until pH is about right. | | |
| 9. 0.8% Agarose gel | 100 ml | | |
| | Agarose | 0.8 | g |
| | in 1xTAE buffer at accurate volume. | | |
| 10. 12% Separating gel | 10 ml | | |
| | Distrilled water | 3.3 | ml |
| | 30 % Acrylamide mix | 0.5 | ml |
| | 1.5 M Tris-HCl, pH 8.8 | 2.5 | ml |
| | 10 % SDS | 0.1 | ml |
| | 10% Ammonium persulfate | 0.1 | ml |
| | TEMED | 0.004 | ml |
| 11. 5% Stacking gel | 3 ml | | |
| | Distrilled water | 2.1 | ml |
| | 30 % Acrylamide mix | 0.5 | ml |
| | 1.5 M Tris-HCl, pH 8.8 | 0.38 | ml |
| | 10 % SDS | 0.03 | ml |
| | 10% Ammonium persulfate | 0.03 | ml |
| | TEMED | 0.004 | ml |

13. Bradford reagent 1 L

| | | |
|---|---|---|
| Coomasive brilliant | 100 | mg |
| 95% ethanol | 50 | ml |
| 85% phosphoric acid | 100 | ml |

The reagent was filtered through Whatman No.1 filter paper to remove impurities and it was stored in coloured reagent bottle at 4°C.

## Appendix C

## Calculation

1. Lignin-modifying enzymes assays

Lignin peroxidase, manganese peroxidase and laccase activities was calculated by using the same equation as follow

$$\text{Enzyme activity} = \frac{\text{Absorbance of enzyme solution x Standard factor}}{\text{Time of incubation (min)}}$$

$$\text{Where as} \quad \text{Standard factor} = \frac{\text{Concentration } (\mu\text{mol /ml })}{\text{Absorbance at 310 nm}}$$

$$A = \varepsilon \times l \times c$$

Where A = Absorbance

$\varepsilon$ = coefficient; $\varepsilon_{310} = 9300\ M^{-1} cm^{-1}$)

c = concentration

l = path length

1.1 Lignin peroxidase assay (Tien and Kirk, 1988)

A total of 1 ml lignin peroxidase assay mixture contained

| | | |
|---|---|---|
| Enzyme solution | 0.05 | ml |
| 4 mM 3,4-dimethoxybenzyl alcohol (veratryl alcohol) | 0.10 | ml |
| 0.2 M sodium tartrate buffer (pH 3.0) | 0.80 | ml |
| 10 mM $H_20_2$ | 0.05 | ml |

The reaction was started by addition of $H_20_2$

One unit of LiP activity was defined as the amount of veratraldehyde ($\mu$mol) released per mL enzyme solution per min in absorbance a 310 nm ($\varepsilon_{310} = 9300\ M^{-1} cm^{-1}$)

1.2 Manganese peroxidase assay (Watanabe et al., 2001)

A total of 1 ml Manganese peroxidase assay mixture contained

| | | |
|---|---|---|
| Enzyme solution | 0.01 | ml |
| 4 mM 2, 6 Dimethoxy phenol | 0.05 | ml |
| 5 mM $MnSO_4$ | 0.10 | ml |
| 0.1 M sodium tartrate buffer (pH 5.0) | 0.25 | ml |
| 0.1 mM $H_20_2$ | 0.1 0 | ml |

The reaction was started by addition of $H_20_2$

One unit of enzyme activity was defined as the increase in absorbance at 465 nm (the oxidation rate of $Mn^{2+}$ to $Mn^{3+}$) per minute (ε= 12.1 x 103 M"1 cm 1)

1.3 Laccase assay (Madhavi and Lele, 2006).

A total of 1.2 ml laccase assay mixture contained

| | | |
|---|---|---|
| Enzyme solution | 0.4 | ml |
| 1 mM ABTS | 0.2 | ml |
| 50 mM sodium succinate buffer (pH 4.5) | 0.2 | ml |

One unit was defined as 1 μM of the increase in absorbance at 436 nm (ABTS oxidization) per minute (ε = 3.6 x 104 $M^{-1}$ cm')

# Appendix E

## Sequences

1. ITS sequence results

ITS4 Reverse primer: TCCTCCGCTTATTGATATGC

ITS5 Forward primer: GGAAGTAAAAGTCGTAACAAGG

Kan2

CCTGATTGAGGTCAGATTGTCAGATAATTGTCTGCAAGAGACTATTGTAAGCACGAATACAAAATACTTCAACACCACAG
CGCAGATAATTATCACACTGAAGGCGATCCGTAACATTCACGCTAATGCATTTAAGAGGAGTCGACTGACTAGAGCCGAC
ACGACCTCCAAGTCCAARTCCACCAGACTTCATTACAAAATCTAGGGATTGAGAATTCCATGACACTCAAACAGGCATGC
TCCGAGGAATACCAAGGAGCGCAAGGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGC
ATTTCGCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTTGCTGAAAGTTGTATATATTGTGTTATACACAGTAAA
CATTCTATAACTGAAGCGTTTGTAATAAAACATAAGAAAGGCTTTCACCAACCGATTAAAGTCGGCTTCGACCATTTCTT
ACTATAAGTTCACAGAGGTTGAGGAGTGGATGAGCCAGGCGTGCACAATGCCTTGTGAAAGGCCAGCGACAACCCGTTCA
AAAACTCGATAATGTCTTCCGCAGTCCTCAG

Skb1

GCTTAAGTTCAGCGGGTAGTCCTACCTGATTTGAGGTCAGAGGTCAATGTGTTGTCTCATAAGAGACGATTAGAAGCTCG
CCAAACGCTTCACGGTCGCGGCGTAGACAATTATCACACCGAGAGCCGATCCGCAAGGAATCAAGCTAATGCATTTGAGA
GGAGCCGACCGTCAGGCCGACAAGCCTCCAAATCCAAGCCTACAAACCCGCAAAGGTTTATAGGTTGAAGATTTCATGAC
ACTCAAACAGGCATGCTCCTCGGAATACCAAGGAGCGCAAGGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAAT
TCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTTGCTGAAAGTTGTATATAGAT
GCGTTATATCGCAATACACATTCATTTACTTGTAAGAGTTTGTAATAAACGCAGGCACAGACGCTTTCACGAACCCGTAA
AGGCTCGCTACACCGTCTGAAACCCACAGTAAGTGCACAGGTGTAGAGTGGATGAGCAGGGCGTGCACATGCCTCGGAAG
GCCAGCTACAACCCAGTCAAAACTCGATAATGATCCTTCCGCAGGTTCACCTACGGAAACCT

Nan6

GCTAAGTTCAGCGGGTAGTCCTACCTGATTTGAGCTCAGAGTTCAGAAATGTTGTCCGAAGACGATTAGAAGCGTGAACA
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GTCGACTACGAGAGCCGACACGACCTCCAAGTCCAAGCCTTCATCAATAAAGTTGAAGGTTGAGAATTCCATGAGACTCA
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ACCTGGCGTGCGCGAACGCACCACCTGGCGTGCGCAAACGCACCACCTACAAGCCGGCCTATGAAAAGTGCACAGAAGTT
GAGTGTGGATGAGCCAGGCGTGCACATACTCTTGCGAGTCAGCAGACAACCCGTTCAAAAACTCGATAATGATCCTTCCG
CAGGTTCACCTACGGAAACC

Kan4

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TATTGTATGCATTCATGCACAGTAAACATTCTATGACAAACAAGTGTATGTAGTAACACACAGACCAGAATTCTTCACCC
GAAGGCTCAAATTCCGATCCATGAGAAGTGCACAGAGGTGTGAAGGTTGGACGAGTGAGGCGTGCACATGCCCTGTTTCC
AAGGCCAGCGACAACCCCTCCCGGTTCATTCAGTAATGATCcTTCCGCAGGTTCACCTACAGAAACCTGT

Sk13

GCTTAAGTTCAGCGGGTAGTCCTACCTGATTTGAGGTCAGAGGTCAATGTGTTGTCTCATAAGAGACGATTAGAAGCTCG
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GGAGCCGACCGTCAGGCCGACAAGCCTCCAAATCCAAGCCTACAAACCCGCAAAGGTTTATAGGTTGAAGATTTCATGAC
ACTCAAACAGGCATGCTCCTCGGAATACCAAGGAGCGCAAGGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAAT
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GCGTTATATCGCAATACACATTCATTTACTTGTAAGAGTTTGTAATAAACGCAGGCACAGACGCTTTCACGAACCCGTAA
AGGCTCGCTACACCGTCTGAAACCCACAGTAAGTGCACAGGTGTAGAGTGGATGAGCAGGGCGTGCACATGCCTCGGAAG
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Kao2

TTTTGCTTAAGTTCAGCGGGTAGTCCTACCTGATTtGAGGTCaGAGGTCAATGTGTTGTCTCATAAGAGACGATTAGAAG
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GTAAAGGCTCGCTACACCGTCTGAAACCCACAGTAAGTGCACAGGTGTAGAGTGGATGAGCAGGGCGTGCACATGCCTCG
GAAGGCCAGCTACAACCCAGTCAAAACTCGATAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGT

Cut3

TGCTTAAGTTCAGCGGGTAGTCCTACCTGATTTGAGGTCAGATGTCAAAGTAAGTTGTCCAACTTAAGGACGGTTAGAAG
CGCAAGCTTATGTTACTTCACGACCACGGCGCAGATAATTATCACACCGAAGCGATCCGTTACACTCACGCTAATACATT
TAAGAGGAGCCGATTCAACGAGGAACCAGCACGACCTCCAAGTCCATGCCTTCGATAACAAAAGTTATGAAGGATGAGAA
TACCATGACACTCAAACAGGCATGCTCCCCGGAATACCAGGGAGCGCAAGGTGCGTTCAAAGATTCGATGATTCACTGAA
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Kao31

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CTACAACAAGTGCACAGAGGTTGAAGAGTGGATGAGCCAGGCGTGCACATGCCCCGAGAGGCCAGCTACAACCTGTTCAG
TTACTCGTTAATGTCCTTCCGCAGTCCT

Sk7

CTACCTGATTTGAGGTCAGATGTCAAAGTAAGTTGTCCAACTTAAGGACGGTTAGAAGCGCAAGCTTATGTTACTTCACG
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TGCTCCCCGGAATACCAGGGAGCGCAAGGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTAT
CGCATTTCGCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTTGCTGAAAGTTGTATAAAGATGCGTTATACGCAA
GGTTACATTCTTAAACTGAAGCGTTTGTAGTAAAACATAGGAAGGCTTCCAAGCGAGCTGTTAGACTCGCCCTTCTGCCG
ACCTACAACAAGTGCACAGAGGTTGAAGAGTGGATGAGCCAGGTGTGCACATGCCCCGAGAGGCCAGCTACAACCCGTTC
AGTTACTCGTTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGT

Kan7

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TTCTTAAACTGAAGCGTTTGTAGTAAAACATAGGAAGGCTTCCAAGCAAAGCATCAGAGGATGCTCGCTCTTCTACCGAC
CTACAACAAGTGCACAGAGGTTGAAGAGTGGATGAGCCAGGCGTGCACATGCCCCGAGAGGCCAGCTACAACCTGTTCAG
TTACTCGTTAATGTCCTTCCGCAGTCCTCAG

Kan1

CCTGATTGAGGTCAGATTGTCAGATAATTGTCTGCAAGAGACTATTGTAAGCACGAATACAAAATACTTCAACACCACAG
CGCAGATAATTATCACACTGAAGGCGATCCGTAACATTCACGCTAATGCATTTAAGAGGAGTCGACTGACTAGAGCCGAC
ACGACCTCCAAGTCCAAGTCCACTAGACTTCATTACAAAATCTAGGGATTGAGAATTCCATGACACTCAAACAGGCATGC
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AAAACTCGATAATGTCTTCCGCAGTCCTAG

Roi2

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CAGAGGAGTCGACTAGAAGCCGACACAACCTCCAAGTCCAAGCCCACTAAACTTCATTACAAAATTTAGGGGTTGAGAAT
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Kan10

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GAAAGTTGTATATAATTGTGTTATACACAGTAAACATTCTATAACTGAAGTGTTTGTGGTAAAACGCAAGAGAAGGCTTG
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TGCACATACCCCAAAAAGAGGTCAGCTACAACCCGTTCAAAACTCGATAATGATCCTTCCGCAGGTTCACCTACAGAAAC
CTTGT

2. Large subunit sequences

LR7 Reverse primer: TACTACCACCAAGATCT

LROR Forward primer: ACCCGCTGAACTTAAGC

Kan10

AATTAAGCATATCAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGCTC
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GACCGCAGGAAAAAGGCCAGGGAAATGTGGCACCCTCGGGTGTGTTATAGTCTCTGGTCATATACTGTGATTGGGACTG
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CCCTGAAAATGGATGGCGCTCAAGCGTGTTACCCATACCTCACCGTCAGTGTTTAAGTGATGCACTGACGAGTAGGCAG
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Skb1

GGAAAGACTACAGGATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGCTCAAATTTAAAATCTGGCGGTCTTTGGCCG
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GAATCCCGTCTTTGACACGGACTACCAGTGCTTTGTGATGCGCTCTCAAAGAGTCGAGTTGTTTGGGAATGCAGCTCAA
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AAAATGGATGGCGCTCAAGCGTGTTACCCATACCTCGCCGTCAGCGTTTAAGTGATGCGCTGACGAGTAGGCAGGCGTG
GAGGTCAGTGAAGAAGCCTAGGCAGTGATGCTGGGTGAAACGGCCTCTAGTGCAGATCT

Cut3

GGAAAGACTACAGGATTCCCCTAGTAACTGCGAGTGAACCGGGAAAAGCTCAAATTTAAAATCTGGCAGCCTTGGCTGT
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CAGCAGGACGGTGGCCATGGAAGTCGGAATCCGCTAAGGAGTGTGTAACAACTCACCTGCCGAATGAACTAGCCCTGAA
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GGTTTGTGAAGAAGCCTAGGCAGTAATGCTGGGTGAAACAGCCTCTAGTGCAGAC



Kao2

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GAATCCCGTCTTTGACACGGACTACCAGTGCTTTGTGATGCGCTCTCAAAGAGTCGAGTTGTTTGGGAATGCAGCTCAA
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Kan1

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Nan6

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Kan4

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Roi2

CCCCAATCTGCACTAGAGGCTGTTTCACCCAGCATTACTGCCTAGGCTTCTTCACAAACCTCCACGCCTGCCTACTCGT
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GGTCCAACGCGGAAAACACTTCTCTAGGATACAACTCGGACGACCAAAGCCGCCAGATTTTAAATTTGAGCTTTTCCCG
CTTCACTCGCAGTTACTAGGGGAATCCTTGTTAGTTTCTTTTCCTCCGCTTATTGATATGCTTAA

3. Lignin peroxidase cDNA

Primers designed for RACE-PCR

3' RACE GCGAGCACAGAATTAATACGACT

3' NESTED RACECGCGGATCCGAATTAATACGACTCACTATAGGT

5' NESTED RACECGCGGATCCGAACACTGCGTTTGCTGGCTTTGATGAAA

5' Inner primer TCGAGACCGATGTTCGGGTGGAA

3' Inner primer CGAGTGGCAGTCCTTCGTCAACAA

CCTCTTCAACGGCGGCGAATGCGGTGCTGAGGCCCACGAGTCCCTCCGCCTCGTCTTCCATGACTCGATCGCCATC
TCGCCCGCTCTGGAGGCCCAGGGCAAGTTCGGTGGCGGCGGCGCCGACGGCTCCATCATGGTCTTTGACGAGATC
GAGACCAACTTCCACCCGAACATCGGTCTCGACGAGGTCGTCAAGCTGCAGAAGCCCTTCGTCCAGAAGCACGGT
GTCACCCCCGGTGACTTCATCGCCTTCGCCGGCGCGGTCGCGCTCTCCAACTGCCCTGGTGCTCCCCAGATGAATT
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CCTTCGTCAACAACCAGAGCAAGCTCGTGTCCGACTTCCAGTTCATCTTCCACTTCCACGACGAGACAGGCGACTCG
CACCGCGTCTACACGTACAGCGTCGGCGAGCACTTCGTCAGGTATAACTCTGACAAGCCCACCATCGTCTACGTCA
CGGGCTCTTAGAGAATTTTTCTACTCGTATTTTCCGCTCCCCAATCCCCAACAACGTGGCTCCCATTCGAATCTTGCA
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GTCGAGTGACTCGAGTCTCCCTCTCCCTTGTGATGTCTCCGTTATCCTGACGTTGCATAGTTTCAGCCCTCCTTGCCC
ACTCTGTGTACTTCGTAGTGTGATTGGATACAAATACTTATCAACTTGATCACG

3. Amino acid sequence of lignin peroxidase from *P. sordida* Sk7

MAFKQLFAAISVVLSLTAAQAAVVKEKRATCSNGKTVSDASCCAWFDVLDDIQQNLFNGGECGAEAHESLRLVFHDSIAISP
ALEAQGKFGGGGADGSIMVFFDEITNFHPNIGLDEVVKLQKPFVQKHGVTPGDFIAFAGAVALSNCPGAPQMNFFTGRAPA
QPAPDQLVPEPFHTVDQIIARVNDAGEFDELELVWMLSAHSVAAVNDVDPTVQGLPFDSTPGVFDSQFFVETQLRGVLFPGS
GGNQGEVESGVPGGIRLQTDHTLARDSRTACEWQSFVNNQSKLVSDFQFIFHFHDETGDSHRVYTYSVGEHFVRYNSDKPT
IVYVTGSRIFLLVFSAPQSPTTWLPFESSCTDQKILHRPIYLGSLDCTTPPVVPQRRRVTRVSLSLVMSPLSRCIVSALLAHSVYF
VVLDTNTYQLDHE

# VITA

Miss Ponlada Permpornsakul was born in Bangkok, Thailand, on July 12, 1985. She graduated from Triam Udom Suksa Pattanakarn School in March 2002 and received the Bacholor of Science degree with a major in Genetics from Chulalongkorn University in April 2006. Since November 2007, she has studied the eree of Philosophy of Science in Biological Sciences Program at Faculty of Science, Chulalongkorn University.