ISOLATION AND SCREENING OF PAHs DEGRADING WHITE-ROT FUNGI

GUNN PANPRAYUN

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ISOLATION AND SCREENING OF PAHs DEGRADING WHITE-ROT FUNGI

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ABSTRACT

This study is concerned with white-rot fungi isolation and screening processes. The fungal collections were done in several parts of Thailand. The samples, such as basidiocarp, pieces of dead and live tree that had been attacked by white-rot fungi, were collected for isolation and screening for potent PAHs degrading strains. The qualitative screening was performed in agar plates using polymeric dye as an indicator. The fungi strains with observed clear zone were used for comparison of their dye decolorizing activity to that of the reference strain, *Trametes versicolor*. The fungi with similar or higher dye decolorizing activity than that of the reference were selected for enzyme assays with Lignin peroxidase, Laccase, and manganese peroxidase, and for benzo[*a*]pyrene degradation. The goal was to isolate the strains of white-rot fungi, which were good degraders of contaminants.

109 strains of white-rot fungi were isolated. However, only 26 strains produced clear zones in the qualitative assay. In the quantitative assay 10 fungi were selected, 7 strains showed a level of decolorizing activity comparable to that of the reference, while 3 strains showed significantly higher activities. For the benzo[*a*]pyrene degradation, K18 showed the highest degrading activity at 39.85%. The enzyme assays showed that K18 had high level of Lignin peroxidase, Laccase, and manganese peroxidase, which indicates that K18 has good potential as a degrader of PAHs. K18 was later identified as *Phanerochaete* sp.

KEY WORDS: PAHs/ DEGRADATION/ LMEs/ WHITE-ROT FUNGI
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การแยกและคัดเลือกเชื้อราประเภทไวท์รอทที่มีความสามารถในการย่อยสลายสารประกอบอะโรมาติกไฮโดรคาร์บอน (ISOLATION AND SCREENING OF PAHs DEGRADING WHITE-ROT FUNGI)

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วท.ม. (เทคโนโลยีที่เหมาะสมเพื่อการพัฒนาทรัพยากรและสิ่งแวดล้อม)

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บทคัดย่อ

งานวิจัยนี้เป็นการแยกและคัดเลือกเชื้อราประเภทไวท์รอทจากแหล่งธรรมชาติที่มีความสามารถในการย่อยสลายสารประกอบอะโรมาติกไฮโดรคาร์บอน โดยทำการแยกเชื้อราประเภทไวท์รอทจากตัวอย่างเศษไม้และเบสิดิโอคาร์พจากพื้นที่ต่างๆในประเทศไทย และทำการคัดเลือกเชื้อที่มีศักยภาพในการย่อยสลายสารประกอบอะโรมาติกไฮโดนคาร์บอน โดยพิจารณาจากความสามารถในการย่อยสีย้อมโพลีอาร์-478 โดยเปรียบเทียบกับเชื้ออ้างอิง Trametes versicolor เมื่อได้สายพันธุ์ต่างๆมาแล้วทำการวัดกิจกรรมของเอนไซม์ชนิดต่างๆของสายพันธุ์ที่ทำการเลือกมาได้แก่ ลิกนินเปอร์ออกซิเดส เลคเคส และ แมงกานิสเปอร์ออกซิเดส รวมทั้งตรวจสอบความสามารถในการย่อยสลายสารประกอบอะโรมาติกไฮโดรคาร์บอน ได้แก่ เบนโซ(*เอ*)ไพรีน โดยผลที่คาดว่าจะได้รับคือ สามารถแยกเชื้อราประเภทไวท์รอทที่มีความสามารถในการย่อยสลายสารปนเปื้อนได้ดี

จากการทดลองพบว่า สามารถแยกเชื้อราประเภทไวท์รอทจากธรรมชาติได้ทั้งหมด 109 ชนิด เมื่อทำการคัดเลือกโดยการย่อยสีโพลีอาร์-478 ในอาหารแข็งพบว่ามี 26สายพันธุ์ที่สร้างเคลียร์โซน และจากการทดสอบกิจกรรมการย่อยสีโพลีอาร์-478 ในอาหารเหลวพบว่ามี 7สายพันธุ์ที่มีกิจกรรมระดับเดียวกับเชื้ออ้างอิง และ 3สายพันธุ์ที่มีกิจกรรมสูงกว่าเชื้ออ้างอิง เมื่อนำเชื้อทั้ง 10 สายพันธุ์ไปตรวจสอบการย่อยสลาย เบนโซ(*เอ*)ไพรีน พบว่าสายพันธุ์ K18 มีความสามารถดีที่สุดโดยสามารถย่อยสลายเบนโซ(*เอ*)ไพรีนได้ 39.85% เมื่อนำสายพันธุ์ K18 ไปตรวจสอบสายพันธุ์ภาพหลังพบว่าเป็นสายพันธุ์ Phanerochaete sp.

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CHAPTER I
INTRODUCTION

1.1 Statement of the problem

Polycyclic aromatic hydrocarbons (PAHs), benzene homologous form from the fusion of three or more benzene rings, are found in considerable number. These arise from natural oil deposits, vegetation decomposition, using of fossil fuel, wood burning, runoff from bitumen roads, waste incineration and industrial process (1). Many of them are produced to reach some human purposes such as 2,4,6-trinitrotoluene (TNT), dichlro-diphenyl-tri-chroethane (DDT), polychlorinated biphenyls (PCBs), synthetic dyes, bleach plant effluent, plastics, organic wood preservatives creosote pentachlorophenol (PCP), and etc. These compounds are very toxic and very persistent in the environment (2).

White-rot fungi are the only group of organism capable of significant aromatic mineralization (1). Several studies have shown that diverse white-rot fungi are capable of PAH mineralization. These include *Phanerochaete chrysosporium* (2), *Pleurotus* sp. (2), *Trametes versicolor* (2), and *Irpex lacteus* (3).

The name white-rot fungi derives from appearance of wood attacked by these fungi, in which lignin removal results in a bleached appearance of the substrate (4). Degradation initiates by one or more of three extracellular enzymes that essential for lignin degradation, and which combine with other processes to effect lignin mineralization. They are often referred to as lignin-modifying enzymes or LMEs. The three enzymes comprise two glycosylated heme-containing peroxidase, ligninperoxidase (LiP, E.C. 1.11.1.14) and Manganese dependant peroxidase (MnP, E.C. 1.11.1.13), and a copper-containing phenoloxidase, laccase (Lac, E.C. 1.10.3.2) (2).

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In the presence of H_2O_2, LiP catalyzes oxidation of an endogenously generated low-molecular-mass redox mediator veratyl alcohol (5), which in turn carries out an one-electron oxidation of non-phenoloic aromatic nuclei in lignin to generate aryl cation radicals. These then degrade non-enzymatically to aromatic and aliphatic products, which are mineralized intracellular. The radicals generated can carry out a variety of reactions, including benzyl alcohol oxidation, carbon-carbon bond cleavage, hydroxylation, phenol dimerization/polymerization, and demethylation. MnP catalyzes an H_2O_2-dependent oxidation of Mn^{2+} to Mn^{3+} and this oxidizes phenolic components of lignin (6). Lac also generates radicals from a low-molecular-mass redox mediator, but in an H_2O_2-independent reaction (2). The LMEs are highly nonspecific with regard to their substrate range. It is unsurprising that white-rot fungi have ability to degrade lignin-like compounds such as PAHs, TNT, PCBs, Organochlorine, wood preservative compounds, plastic, and synthetic dye. When their mode of action, which involves generation of free radicals, is considered.

White-rot fungi such as *Phanerochaete chrysosporium*, *Phanerochaete sordida*, *Trametes versicolor*, *Trametes cigulata*, *Cyathus stercoreus*, *Coriolopsis polyzona*, and *Pleurotus sajor caju* are well known as high level producers of LMEs. Therefore, they have been widely used in decomposition studies of aromatic compounds (1). In Thailand, only *T. versicolor* has been isolated and made available for public use. Thus, the purpose of this study is to obtain novel strains of white-rot fungi with high activity in degrading aromatic compounds from natural environment of Thailand. Due to ability to degrade synthetic dyes, Poly R-478 (7) was chosen for screening of the required white-rot fungi. The enzyme assays were performed to measure volumes of LMEs. The selected strains were later tested for ability to degrade PAH using PAH degrading assay. As a representative for PAHs, benzo[*a*]pyrene was chosen for the purpose. The strains of white-rot fungi that could significantly degraded benzo[*a*]pyrene were selected and identified.

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1.2 Objective

The objective of this study is to isolate the strains of high LMEs producing white-rot fungi, which could degrade benzo[*a*]pyrene at least as high as *T. versicolor*, from natural environment of Thailand.

1.3 Conceptual Framework

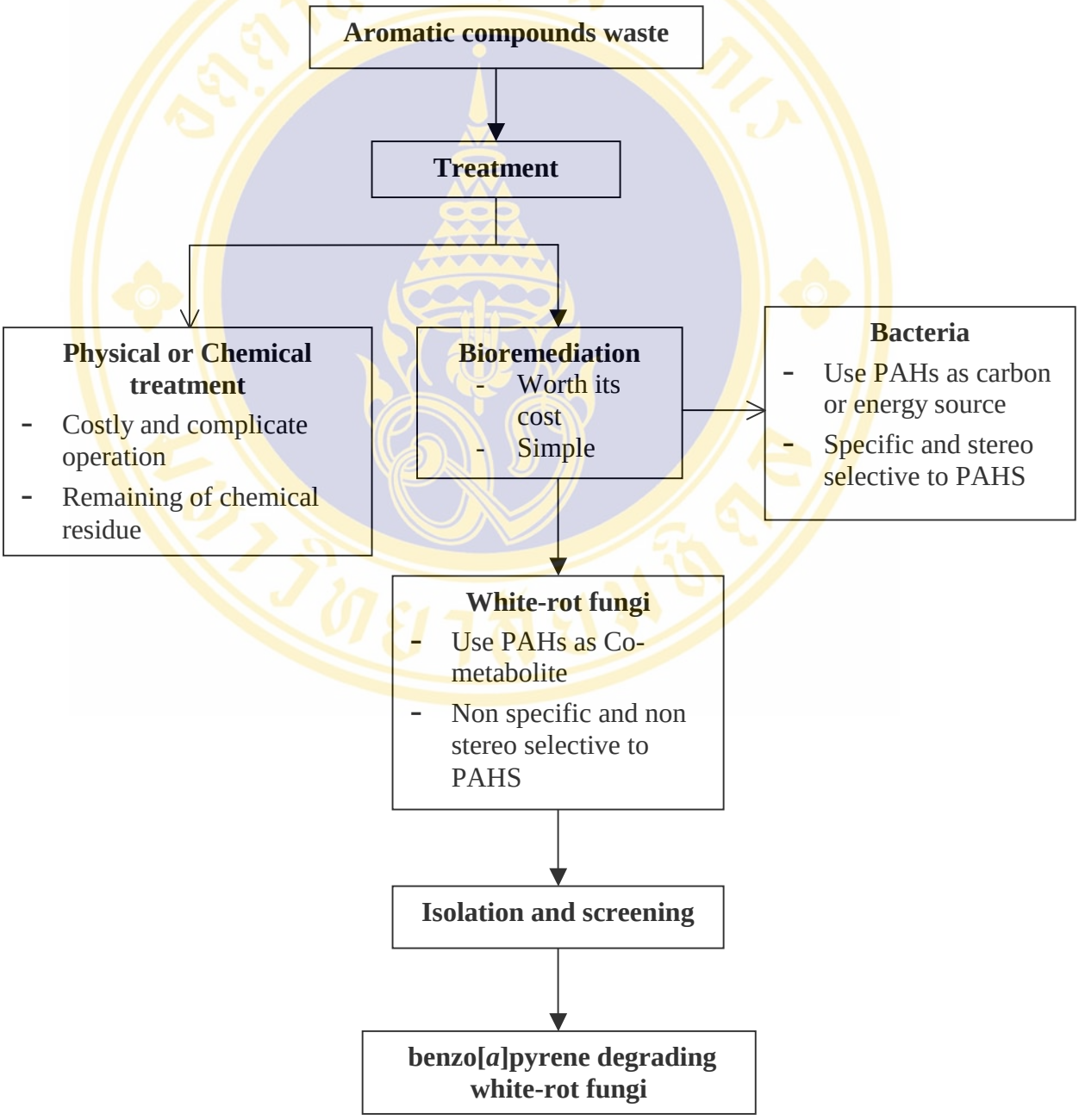

Figure 1.1 Conceptual framework

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1.4 Scope

1. Collection of basidiocarps or pieces of rotten wood were conducted in several forests is all parts of Thailand.
2. Clear zone observation and absorbance measurement were used for determination of poly R-478 degradation.
3. Selected dye degrading strains were chosen for the enzyme assays
4. There LMEs assays, including lignin peroxidase, laccase, and manganese peroxidase, were performed.
5. Strains with high dye degrading rate were selected for the benzo[*a*]pyrene degradation test. The measurement of benzo[*a*]pyrene residue in the sample were conducted using HPLC.

1.5 Hypothesis

Diverse gene pool of white-rot fungi in natural environment of Thailand could provide the strains with higher benzo[*a*]pyrene degrading activity than *T. versicolor*.

1.6 Anticipated Benefits

1. High potent white-rot fungi strains for aromatic compounds degradation.
2. A novel bioremediation method for cleaning up benzo[*a*]pyrene polluted environment.

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1.7 Definitions

White-rot fungi: The origin of “white-rot fungi” is from ability to digest cellulose or/and lignin in cell wall of plants, making “white rotten” on the plants. They also have been called “basidiomycetes”. They have been classified to division *Amastigomycota* subdivision *Basidiomycortina*. Most of them belong to class *Hymenomycetes* subclass *Holobasidiomycetidae* order *Aphyllophorales*.

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CHAPTER II
LITERATURE REVIEW

2.1 Aromatic compounds and polycyclic aromatic compounds

Benzene and its derivatives were originally called aromatic because many of the compounds known as early as 19th century had a fragrant odor. These included, vanillin that was isolated from vanilla bean, and methyl salicylate from oil of wintergreen plant. Today, the term aromatic has a completely different meaning; instead of a number of compounds with pleasant odors; it refers to a family of compounds consisting of benzene and its derivatives.

Michael Faraday first isolated benzene in 1825 from compressed illuminating gas, which was used in England as a source of light. By the year 1858, chemists knew a good deal about benzene. The empirical formula of the compound was found to be CH, and when the molecular weight was determined to be 78g/mol, the molecular formula of the C_6H_6 was assigned to the compound. In addition, benzene was found to show the following chemical properties:

1. Benzene formed only one monosubstituted derivative, C_6H_5X. This means that all six hydrogens in benzene are equivalent.
2. Benzene reacted to yield three disubstituted derivatives, C_6H_4XY or $C_6H_4X_2$.
3. One mole of benzene reacted with ozone followed by zinc and water to produce 3 mol of O=CH-HC=O, or glyoxal.

Historically, a significant number of aromatic compounds were known before the superior IUPAC system of nomenclature was introduced. Therefore, it seems reasonable that the use of nonsystematic names to designate aromatic compounds has carried over through the years. In fact, this carryover has been so extreme that several

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of the names of the monosubstituted benzenes have been accepted by the IUPAC as systematical names (Figure 2-1).

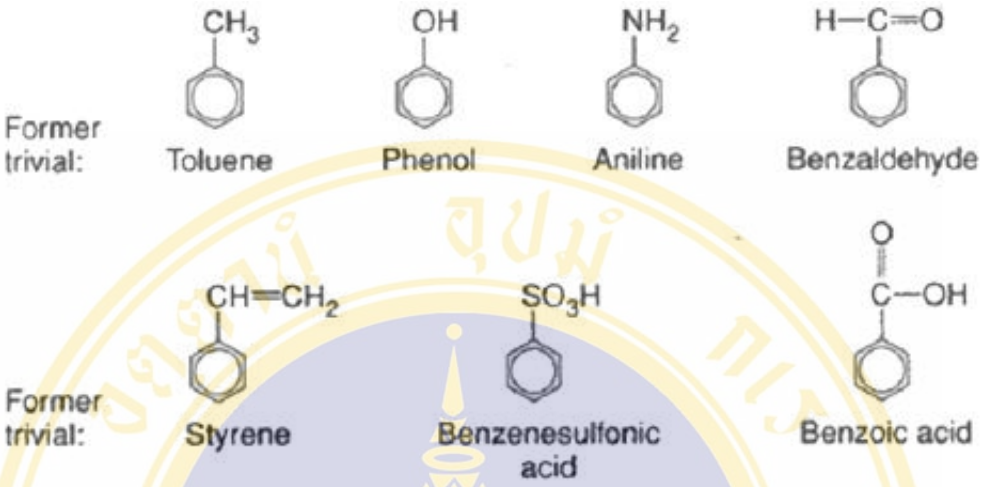

Figure 2-1 Former trivial nomenclature of selected monosubstituted benzene derivatives (8)

2.1.1 Aromatic compounds

2.1.1.1 Monosubstituted Benzenes

These compounds are named as derivatives of benzene, such as nitrobenzene, the halobenzene, and a number of the alkylbenzenes, or arenes. No number is needed to locate a substituent in monosubstituted benzene because the six hydrogens in benzene are equivalent. Some examples of the nomenclature of selected monosubstituted derivatives are shown in Figure 2-2. Practicing scientists tend to use the name toluene rather than methylbenzene, phenol rather than hydrobenzene, etc.

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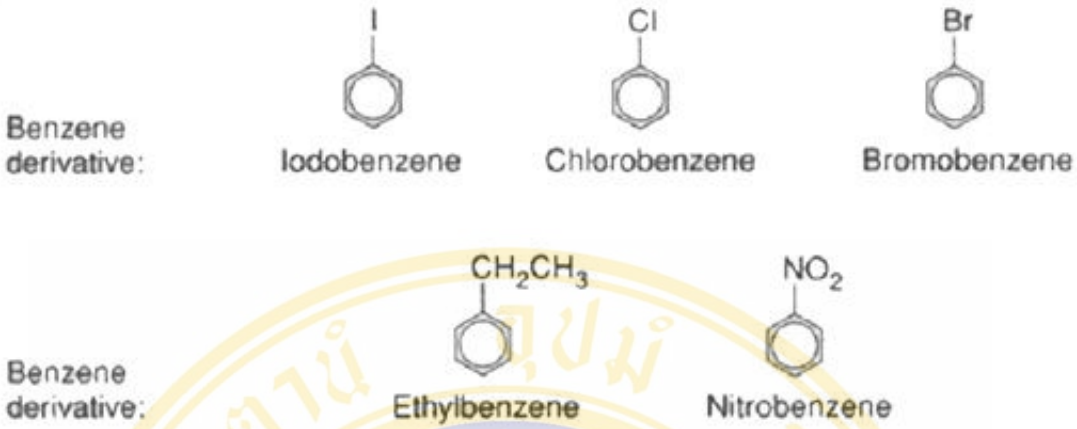

Figure 2-2 Benzene-derivatives nomenclature of selected monosubstituted benzene derivatives (8)

Just as a hydrogen atom is lost by an alkane to give an alkyl group, in the same manner a hydrogen atom can be lost by an aromatic compound to give an aryl group. Two important aryl groups used in nomenclature are shown in Figure 2-3.

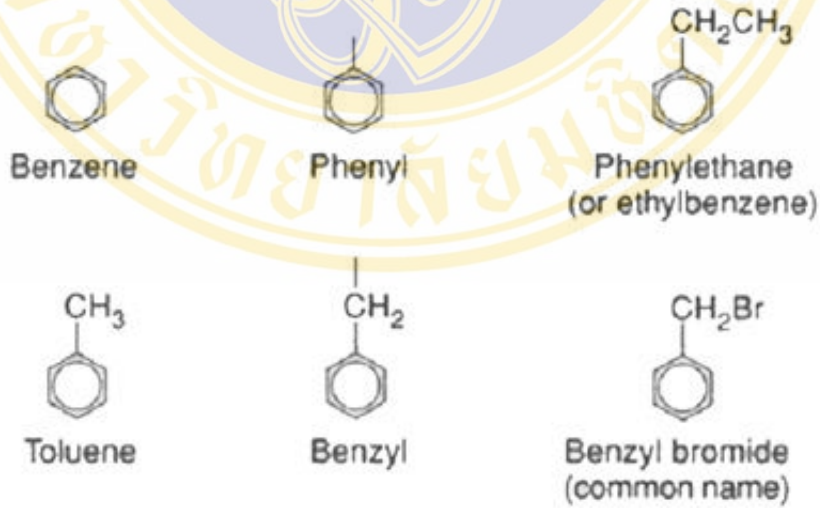

Figure 2-3 Summary of selected aryl groups derived from benzene and toluene (8)

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2.1.1.2 Disubstituted Benzenes

These derivatives are named using the prefixes ortho-, meta-, and para-. The prefixes designated for two groups are bounded to each two position of carbons on a ring, which are given in Figure 2-4.

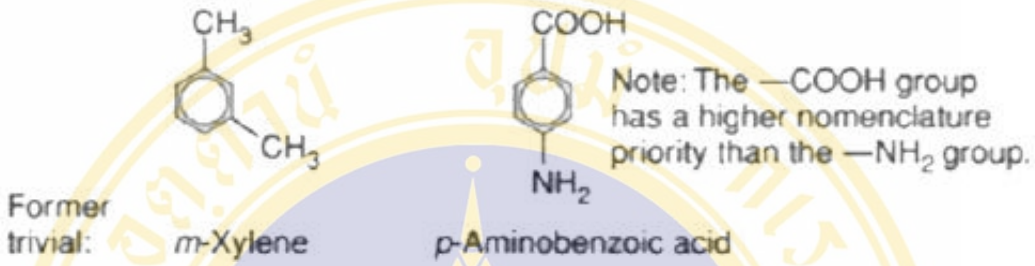

Figure 2-4 Former Trivial nomenclature of selected disubstituted benzene derivatives (8)

2.1.1.3 Benzene derivatives with three or more substituents

These compounds are named by assigning to each substituent the smallest possible carbon number on the ring as shown in Figure 2-5.

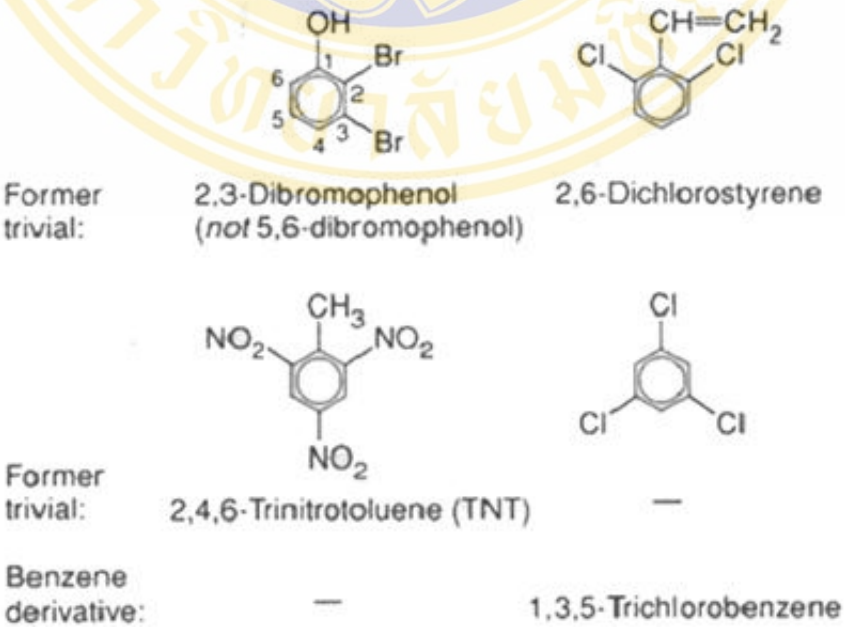

Figure 2-5 Nomenclature of selected trisubstituted and tetrasubstituted benzene derivatives (8)

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2.1.2 Polycyclic aromatic compounds

Polycyclic aromatic hydrocarbons (PAHs) are sometimes referred to as polynuclear aromatic hydrocarbons or as polycyclic aromatic compounds. PAHs (including aromatics) are considered to be the most acutely toxic component of petroleum products, and are also associated with chronic and carcinogenic effects in human (9). Aromatics are often distinguished by the number of rings they possess, which may range from one to five. Aromatics with two or more rings are referred to as PAHs (9). PAHs may or may not have substituent groups attached to one or more rings. For example, Alkyl and chlorine groups attached to PAHs change the fate and effects characteristics, often for the worse. There are more than 100 different PAH compounds (10). Parent compounds of PAHs are given in Figure 2-6.

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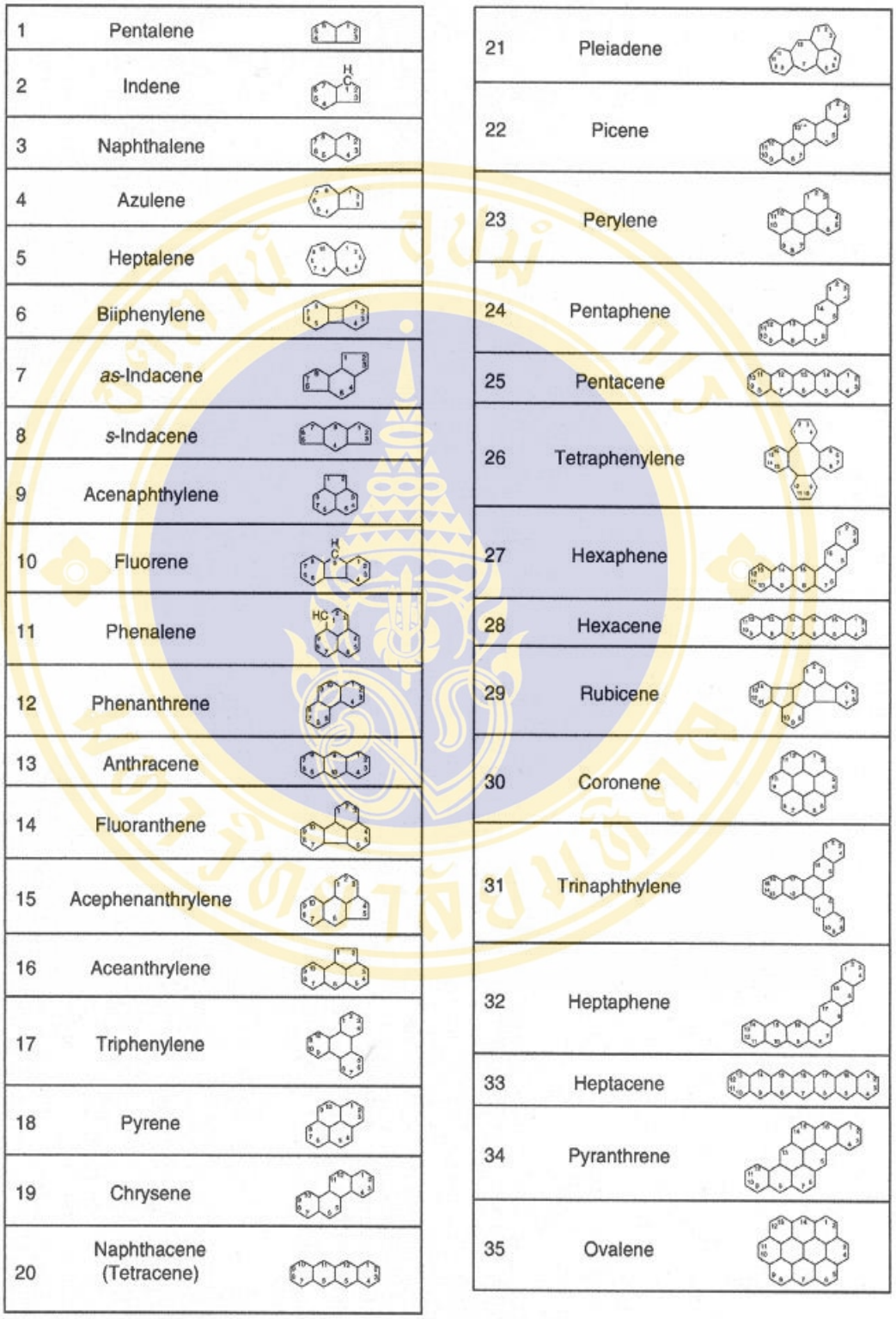

Figure 2-6 Parent PAHs compounds (11)

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PAHs are among organic compounds, which EPA classifies as Base/Neutrals (Extractable) and semi-volatile organics (12). In the strictest definition, PAHs are composed of "two or more fused aromatic (benzene) rings" (13). However, most important criteria in classifying PAHs are the present of two benzene rings in the chemical structure. Therefore, a compound such as biphenyl is considered by most environmental chemists to be a PAH even though the two ring structure is joined directly rather than fused.

In general, PAHs may be divided into two groups, depending upon their physical and chemical properties: low-molecular-weight PAHs, containing three or fewer aromatic rings, and high-molecular-weight PAHs, containing more than three aromatic rings. Hwever, most information on the forms and fate of PAHs in the aquatic environment is available for only a few compounds, specifically naphthalene, anthracene, benz[*a*]anthracene and benzo[*a*]pyrene) (14).

2.1.3 Source of Aromatic hydrocarbons

Two main industrial sources of aromatic hydrocarbons are coal tar and petroleum. Coke and coal tar are obtained by heating coal in the absence of air. If coal tar is distilled, a number of aromatic hydrocarbons could be obtained, including benzene, toluene, dimethylbenzenes (xylenes), naphthalene, and anthracene. Many other hydrocarbons may be obtained as well. Aromatic compounds may also be obtained from petroleum by a process called “reforming” that converts alkanes and cycloalkans into aromatic compounds (8).

Benzene and toluene are used as solvent in organic reactions and as starting materials for many organic syntheses. Naphthalene has long been employed as an insecticide to keep clothing free of moths during the summer season.

A number of PAHs classified as carcinogens can be isolated from cigarette and smoke. One of the most potent of the known carcinogens is 1,2-benzopyrene. The benzo group is a C_6H_4 aromatic unit that is bonded to two adjacent carbons of the pyrene ring (8).

PAHs are among the most hazardous compounds in oil spills (13). PAHs are found in crude oil, used motor oil, incomplete combustion, and in various complex

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mixtures of hazardous chemicals (such as creosote). The main sources of human exposure are food intake and inhalation. Food intake can be an important route for fish and wildlife too, along with direct contact in water, sediment, and soil environments.

2.1.4 Toxicity

Studies in animals have also shown that PAHs can cause harmful effects on skin, body fluids, and immune system after both short- and long-term exposure (15). PAHs have been changed by all tissues in the body into many different substances (15). Some of these substances are more harmful and some are less harmful than the original PAHs (15).

Some studies have concluded that the acute toxicity of PAHs in oil appears to be a function of its two-ring hydrocarbons such as naphthalene, content (16).

Acute toxicity is rarely reported in humans, fish, or wildlife, as a result of exposure to low levels of a single PAH compound. However, PAHs are more frequently associated with chronic risks. These risks include cancer and often are the result of exposures to complex mixtures of chronic-risk aromatics (such as PAHs, alkyl PAHs, benzenes, and alkyl benzenes), rather than exposures to low levels of a single compound (16).

2.1.4.1 Hazards to Fish, Wildlife and other Non-Human Biota

Probably the most important target to analyze in natural resource damage assessments for oil spills is PAHs and the homologous series (alkylated) PAHs (17). Alkylated PAHs are often more abundant, persist for a longer time, and are sometimes more toxic than the parent PAHs (17). For example, methyl phenanthrene is more toxic than the parent compound phenanthrene. Metabolism of PAHs does not necessarily mean a reduction in the biological potency of the compound to harm the organism, since the metabolites are often more hazardous (18, 19).

Hazards from PAHs in the aquatic environment are often difficult to assess due to the great number of PAHs and alkyl PAHs of potential concern and the number of variables that can either increase or decrease the risk. The traditional concern of

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environmental toxicologists related to PAHs has focused on the metabolic breakdown of Benzo[a]pyrene and several other PAHs into metabolites far more carcinogenic and otherwise hazardous than the original parent compounds (19). Total carcinogenic PAHs as low as 1.0 mg/kg have been shown to induce tumors in brown bullhead catfish (20). However, the significance of cancer in fish and other aquatic populations has not been adequately determined (19).

PAHs tend to partition into sediments and soils, with relatively small concentrations of many PAHs showing up in water due to low solubility (19). Except for the more soluble and particularly toxic two-ring compounds such as the naphthalenes (21, 22), water toxicity has historically not been thought to be a big problem for most PAHs. It had been thought that when the PAH molecular weight reaches that of three-ring compounds, an aqueous concentration equal to the solubility in water was required to elicit aquatic toxicity, and that heavier compounds would not exhibit acute toxicity even at solubility concentrations, the maximum concentrations water would hold at ordinary temperatures and pressures (23).

However, recent studies have shown that some of the heavier PAHs can exhibit acute water toxicity at levels below solubility due to photo-enhanced toxicity in the presence of ultraviolet (UV) or other types of solar radiation (23, 24, 25). The common PAH naphthalene is selectively phytotoxic, while alkyl compounds are most toxic (26), especially to benthic aquatic invertebrates and fish. Phototoxicity of PAHs has been seen in a wide variety of aquatic organisms, including aquatic plants (24).

Phototoxicity of PAHs was discovered when organisms, which had survived lab exposures to PAHs, died quickly after being moved into sunlight. An increase in toxicity due to photo-induced changes is called phototoxicity. Tests performed in the presence of UV or other solar radiation showed greatly increased toxicity to those same organisms at PAH concentrations below maximum solubility (19, 24, 25, 27).

One of the reasons that PAHs are a potential risk to aquatic biota is that fuels or crude oils containing PAHs are so often spilled into aquatic environments. For example, heavier and more persistent PAHs are found in gasolines (among other oil products) (28). Although they make up small percentages of gasolines, they are more persistent than most other constituents of gasoline and tend to have greater

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carcinogenic and other chronic impact potential. Thus, although PAHs typically represent a small percentage of the total mass of volume of gasoline spilled, a few months later the PAHs represent a relatively large proportion of the hazardous components which still remain in contaminated soils or sediments (by then the also hazardous but more mobile and volatile BTEX compounds have often migrated into the air or groundwater) (28)

2.1.4.2 Potential Effects on Humans

Many of the same complications for predicting effects of PAH mixtures on humans are true for predictions related to fish and wildlife. The traditional concern of human toxicologists related to PAHs has focused on the metabolic breakdown of benzo[a]pyrene and several other PAHs to metabolites far more carcinogenic and otherwise hazardous than the original parent compounds (19).

Although PAHs usually occur in the presence of other PAHs, the combined effects of mixtures of PAHs upon humans is not well known, the main carcinogens tend to be metabolites rather than parent compounds, and there is very little information on some of the suspect compounds; the bottom line is that precise risk assessments of mixtures of PAHs is a very difficult task. In an effort somewhat similar to the TCDD equivalents method for dioxins, risk assessment researchers are considering assigning relative potency to various PAHs using benzo[a]pyrene (BAP, the most studied PAH) as the standard.

In the environment, humans are most likely to be exposed to PAH vapors or PAHs that are attached to dust and other particles in the air. Sources include cigarette smoke, vehicle exhausts, asphalt roads, coal, coal tar, wildfires, agricultural burning, residential wood burning, municipal and industrial waste incineration, and hazardous waste sites. PAHs can enter humans through human lung by inhaling air that contains them (usually apsorbed on to particles or dust). Cigarette smoke, wood smoke, coal smoke, and smoke from many industrial sites may contain PAHs (15). People living near hazardous waste sites can also be exposed by breathing air containing PAHs (15). Background levels of some representative PAHs in the air are reported to be 0.02-1.2 nanograms per cubic meter in rural areas and 0.15-19.3 ng/m3 in urban areas (15). The

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exposure could also occur near areas where coal, wood, gasoline, or other products have been burned.

In addition, areas near hazardous waste sites, such as former manufactured-gas factory sites and wood-preserving facilities could also be potential sources. PAHs have been found in some drinking water supplies in the United States (15). In the home, PAHs are present in tobacco smoke, from wood fires, creosote-treated wood products, cereals, grains, flour, bread, vegetables, fruits, meat, processed or pickled foods, and contaminated cow's milk or human breast milk (15). Cooking meat or other food at high temperatures, which happens during grilling or charring, increases the amount of PAHs in the food (15).

2.1.4.3 Carcinogenicity

Many PAHs, and several breakdown products of PAHs have been documented to be tumorgenic (20). In general, the heavier (4-, 5-, and 6-ring) PAHs have greater carcinogenic potential than the lighter (2- and 3-ring) PAHs (29). The 4- to 7-ring PAHs have been especially implicated in the carcinogenic effect of used oil (29).

There are many reports on the possibility of cancer in both fish and humans resulting from exposure to complex mixtures of PAHs (13, 15, 17, 20, 21, 23, 29). Correlation between PAH metabolites in the gallbladder or the degree of PAH pollution in sediments has been positively correlated with DNA adducts or hepatic neoplasms in fish (30). EPA has also given PAHs as a group the regulatory classification of "carcinogenic" (31).

However, literature sources vary in classifying various individual PAHs as carcinogens or non-carcinogens. Some glucuronides (metabolic conjugates) are less toxic than the parent compound, but one PAH glucuronide (N-hydroxyacetylaminofluorene glucuronide) is actually a stronger carcinogen than the parent compound N-hydroxyacetylaminofluorene (32).

Different species and individuals also vary in their susceptibility to carcinogens. The modern trend is towards incorporating the route of exposure and species into the statement, for example, stating that the compound is carcinogenic to mice by inhalation but not by ingestion (15).

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Very few alkyl PAHs have been broadly tested for carcinogenicity, but it is known that both dimethylbenzo[*a*]anthracene and its parent compound benzo[*a*]anthracene are carcinogenic (10, 15, 20, 30). Methylbenzo[*a*]anthracene is actually more carcinogenic than its parent compound benzo[*a*]anthracene, and dimethylbenzo[*a*]anthracene is even more carcinogenic (20). Both cholanthrene and its 3-methyl alkyl cholanthrene counterpart are carcinogenic (20, 30). It is also known that alkylation does not significantly change phototoxicity (19, 27) and that there are some relationships between phototoxicity and potential carcinogenicity. Thus, it would not be surprising to discover that a notable number of alkyl PAHs are carcinogenic although they are not now typically added to the list of "carcinogenic PAHs" considered in risk assessments. Naphthalene is an example of a common PAH formerly classified as generally non-carcinogenic by EPA (31) and previously often given a non-carcinogenic rating in risk assessments. It is now classified as non-classifiable (12) with a note in IRIS that it will probably be upgraded to "possibly carcinogenic". Current evidence suggested that naphthalene causes carcinogenicity to mice exposed by inhalation (15). There are also some indications that naphthalene may act as a promoter for lung tumors started by other carcinogens (12). Since naphthalenes often occur in petroleum hydrocarbon mixtures, which contain strong carcinogens, a carcinogenic promoter role may prove environmentally significant.

2.2 **White-rot fungi** (33, 34, 35, 36)

White-rot fungi have been classified in division *Amastigomycota* subdivision *Basidiomycortina*, which contain about 15,000 known species. These fungi could be informally referred as the “basidiomycetes”, a term which is commonly used but without taxonomic status. Many of common names refer to the visible part of the fungus, which is a conspicuous reproductive body, the basidiocarp. The basidiocarp is supported by extensive assimilative mycelium penetrating the soil or plant materials and thus deriving the nutrients that supply the basidiocarp. Most of the white-rot fungi are in class *Hymenomycetes* subclass *Holobasidiomycetidae* order *Aphyllophorales*. The *Aphyllophorales* is a large order containing about 2,000 known species. Many of these are the bracket and coral fungi. The vast majority posing plant remains, as they are able to digest cellulose or lignin that occurs in plant cell walls. Also, many

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members of the *Aphyllophorales* cause destruction of timber and wood products or decay wood in standing tree.

There is a great deal of morphological variation in this group. Those characters unifying the group are the possession of a holobasidium in an exposed hymenium borne on a dry or comparatively dry basidiocarp. The texture of the basidiocarp may be similar to that of cork, wood, leather, paper, or cartilage. Unlike the basidiocarps of the Order *Agaricales*, the basidiocarps of the *Aphyllophorales* are not fleshy and moist.

The simplest basidiocarp forms are crustlike and resupinate, lying appressed to the surface of the substratum and with a hymenium covering the entire exposed surface. Other basidiocarps may be similarly flat and appressed to the substratum but have a portion of the basidiocarp reflected or turned away from the substratum (in these, the hymenium would occur on one surface only). Upright basidiocarps also occur and may assume various forms such as a funnel (with the hymenium on the outer surface) or upright clubs or stalks, which may be branched or not branched. Some of the basidiocarps form a definite cap, the pileus, which bears the hymenium on the lower surface. The pileus may be bilaterally symmetrical, resembling a shell or shelf, and may be attached directly to the substratum at its side or it may be attached by stalk. A few of the species that form a pileus have one that is symmetrical radial, and is supported by a stripe attached to its center.

These basidiocarps range from somewhat fleshy, cartilaginous forms to forms that are hard and woody. The trama is generally comprised of elongate hyphae. Generative hyphae, sometimes with inflated segments or cells, predominate in the softer forms. The hard, woody forms often have skeletal or binding hyphae or both in addition to the generative hyphae. We use the term monomitic to refer to a tissue having only generative hyphae, dimitic for a tissue having generative hyphae and one additional hyphal type (either skeletal or binding hyphae), and trimitic for a tissue with all three hyphal types.

That of the trama immediately beneath it most often determines the form of the hymenium. Because this trama bears the hymenium and determines its shape, it is called the hymenophore. The hymenophore may be smooth or in the form of warts or spines; it may line the interior of tubes or cover the surface of gills (leaf-like lamellae

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that resemble the pages of a half-opened book). In terms of development of the hymenium, two distinct types may be distinguished, the catahymenium and euhymenium. The catahymenium consists at first of a layer of sterile hyphal elements; the developing basidia then push into the catahymenium and gradually extend through and beyond it. In contrast, the euhymenium lacks such a layer, but the first hymenial elements to appear are the basidia intermixed with a few cystidia. Sometimes the euhymenium may become progressively thicker as it ages. If the euhymenium thickens, it will produce successive layers of basidia, with the older basidia and cystidia buried beneath a layer of younger basidia. As each layer of basidia reaches maturity and then disintegrates, it is penertrated by hyphae that arise from the subhymenium and form new basidia on the surface. The euhymenium does not thicken in many species, and if it does, all basidia in it will mature simultaneously and will not be replaced by younger basidia.

Division of the members of the *Aphyllophorales* into genera was originally made on the basis of gross morphology of the basidiocarp and hymenium. In the more modern systems, a complex of characters is used. These include many microscopically and anatomical features, as well as the more conspicuous ones of gross morphology. Some families in this order are shown as following.

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Corticium, probably the simplest basidiocarps are those formed by species of *Corticum*. These are thin, flattened resupinate basidiocarps that are completely appressed to the wood or bark on which they occur. The hymenium is flat and covers the entire surface of the basidiocarps. When fresh, the basidiocarp is somewhat membranous or crusty, but when dry it may crack in a manner similar to the old paint that it resembles. Basidiocarps of species of *Stereum* (Figure 2-7) are similar except that they have a leathery texture and are not completely resupinate but have a recurved edge.

Figure 2-7 Basidiocarp of *Stereum* having a smooth hymenophore (33).

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Clavaria and Allies. Upright basidiocarps with a smooth hymenium occur in members of several genera, including *Typhula* and *Clavaria*. *Typhula* species form a club-shaped unbranched basidiocarp arising from a sclerotium. The outer surface of this basidiocarp is covered by a smooth hymenium, and the texture is fleshy to waxy. Many species of *Clavaria* (Figure 2-8) and allied genera also form unbranched basidiocarps, but this group of the fungi is especially notable for producing repeatedly branched basidiocarps that often resemble antlers or coral, hence their common name, the coral fungi. This branching is often quite regular, and may occur in a dichotomous or symmetrical radial pattern. These fungi are often colorful and may be yellow, red, purple, or violet as well as the more sedate shades of white, cream, gray, or brown. Large specimens may weigh several pounds. These fungi usually form their basidiocarps directly on the ground, usually in humus. The hymenium is smooth and covers virtually the entire basidiocarp. In members of some genera, basidia bear only two basidiospores each. The trama has generative hyphae that may be inflated but lacks binding hyphae. Clamp connections may be present or absent, depending upon the species. Cystidia are frequently present in the hymenium.

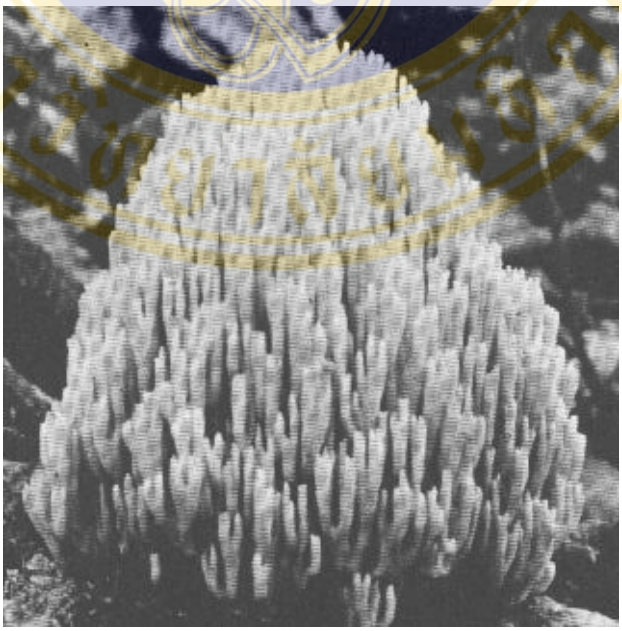

Figure 2-8 Basidocarp of *Clavaria*. The smooth hymenophore covers the upright branches (33).

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Cantharellus and Allies. These fungi form a fleshy basidiocarp that is usually trumpet- or funnel-shape. They are most often some shade of yellow or orange but may occur in other colors and usually occur on the ground in autumn. The hymenium is borne on the lower surface of the pileus but not on the stipe. In members of genus *Craterellus* (Figure 2-9), as well as in some species of *Cantharellus*, the hymenium is smooth. In most species of *Cantharellus*, the hymenium is folded into shallow, rounded ridges that are longitudinally oriented or repeatedly merge and separate from each other in a reticulate pattern.

Figure 2-9 Basidiocarps of *Craterellus taxophilus*. The smooth hymenophore lines outer surface (33).

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Hydnum and Allies. In members of the genus *Hydnum* and its allies, the hymenium covers spines or teeth. There is great variety in the form of the basidiocarps. They may be resupinate and have the hymenium covering the entire surface or have a pileus. The pileus, which has the hymenium on the underside, may be either laterally attached or borne on the stipe. The hedgehog fungus, *Hericium caput-ursi* (Figure 2-10) forms a basidiocarp consisting principally of a fleshy mass of pendant teeth hanging down from a mass of fungal tissue. The size of these tooth fungi varies considerably, as their textures, which may be fleshy, leathery, woody, waxy, or membranous. They are most often drab in color and are usually some shade of brown. The majority occurs as saprophytes on wood, while others occur on the ground in forests.

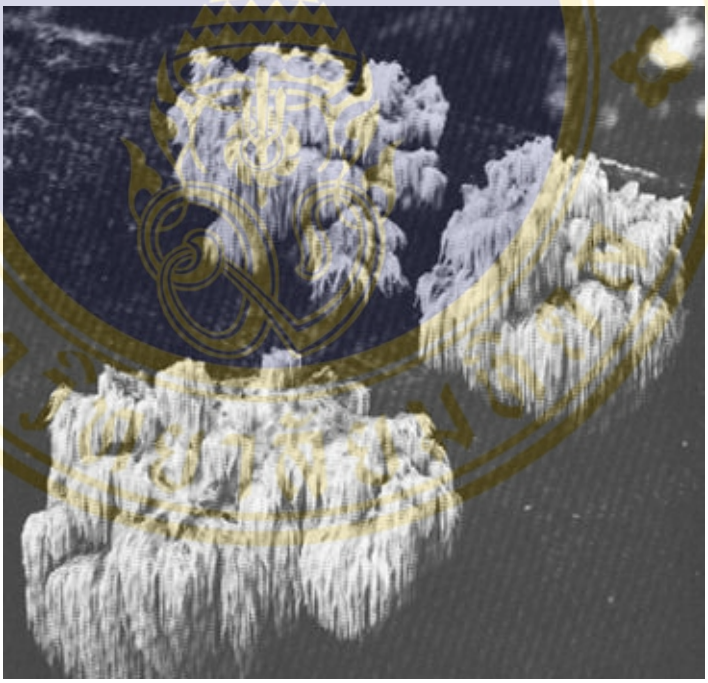

Figure 2.10 The basidiocarps of the hedgehog fungus *Hericium caput-ursi.* The hymenophore covers the downward-hanging teeth (33).

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Polyporus and Allies (*Polypores*). These fungi occur predominantly as saprophytes on dead woody plant parts such as tree trunks, stumps, and branches. A few occur on living trees, where they cause heart rot, and others occur on soil.

Species of *Polyporus* and allies genera form tubes lined with the hymenium. If these tubes are examined from the end one sees a surface punctuated by many pores. This may be easily visualized if one examines a handful of parallel drinking straws from the end. The generic name is derived from this poroid appearance and provides a common name for these fungi, the "polypores." Although the pores are usually circular, they may be elongated and labyrinthine as showed in Figure 2-11, elongated and hexagonal, or elongated and most gill-like.

The basidiocarps may be fleshy when they are young, but become dry and like wood, cork, or leather as they mature. The basidiocarps are often quite large, sometimes reaching 25 centimeters or more in diameter. Some of the basidiocarps are resupinate and have the hymenial tubes covering the surface as showed in Figure 2-12. Others form a pileus that is attached by broad surface to the substratum and thus resemble a shelf. These shelf-like fungi are often called "bracket fungi." Still other basidiocarps may have a stalk, extending from either the side or the center of the pileus. The majority of the fungi are annual and the mycelium produces new basidiocarps every year.

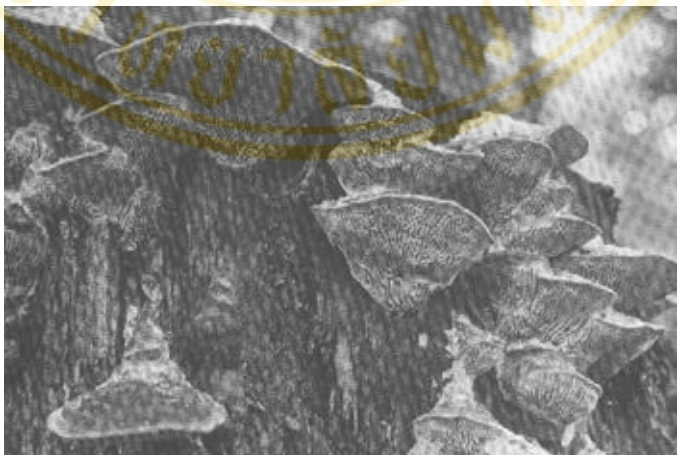

Figure 2-11 Basidiocarps of the braket fungus *Daedalea confragosa.* (33).

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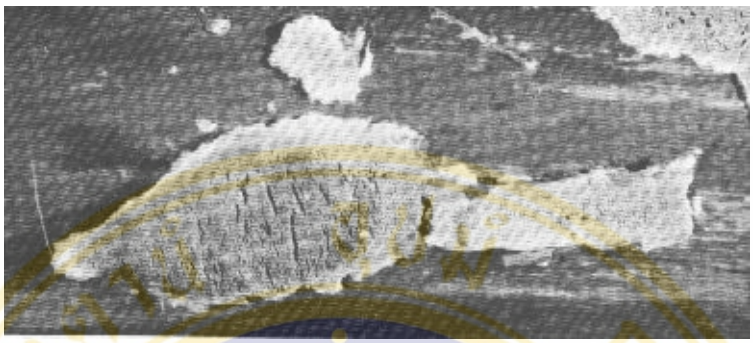

Figure 2-12 A resupinate basidiocarp of *Poria europa* (33).

Species of *Fomes*, however, are perennial and the laterally attached basidiocarps are viable for many years. In these perennial polypores, a new layer of tubes develops each year. The new layer of tubes is formed beneath the one that was produced the previous season, and only the most recently formed layer of tubes produces basidiospores. The basidiocarps are usually cream, gray, brown, or black in color but are sometimes shades of yellow, orange, or red. In those species forming a pileus, the color of the upper, sterile surface may be the same or different from that of the tubular region.

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2.3 The ligninolytic Enzymes system

Lignin is a major component of plant material and the most abundant form of aromatic carbon in the biosphere providing rigidity to all higher plants by acting as glue between the cellulose fibers. In addition, Lignin forms a barrier against microbial destruction and protects the readily degradable carbohydrates (cellulose, hemicellulose). From the chemical point of view, lignin is a heterogeneous, optically inactive polymer consisting of phenylpropanoid interunits, which are linked by several covalent bonds, aryl-ether, aryl-aryl, carbon-carbon bonds (35) (lignin monomers are given in Figure 2-13). The polymer arises from enzyme-initiated polymerization of phenolic precursors via the radical coupling of their corresponding phenoxy radicals. Due to type of bonds and their heterogenity, lignin cannot be degraded by hydrolytic mechanism as most other natural polymers can. Within the course of evolution, only certain basidiomycetous fungi have developed an efficient enzyme system to degrade lignin so that these microorganisms play an important role in maintaining the global carbon cycle (36). Due to their ability to selectively remove lignin, while leaving white cellulose fibers, these fungi are also called white-rot fungi. Typical representatives of these fungi are the wood degrader *Trametes versicolor*, *Phanerochaete chrysosporium, Pleurotus ostretus*, and *Nematoloma frowardii*. Lignin degradation does not provide a primary source of carbon and energy for fungal growth and is, therefore, a general co-metabolic process (hemicelluloses are mostly used as growth substrate).

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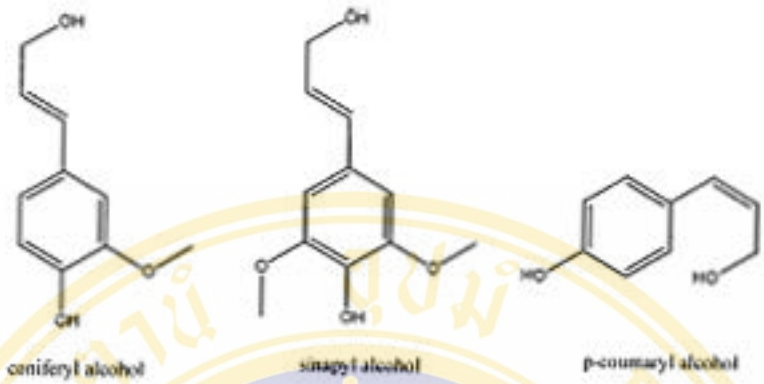

Figure 2-13 Lignin monomers (2)

Lignin degradation is very probably brought about by the synergistic cooperation of different ligninolytic enzymes. Three phenol-oxidizing enzymes, Manganese peroxidase (MnP) or E.C. 1.11.1.13; Mn(II):H_2O_2 oxidoreductase, Lignin peroxidase (LiP) or E.C. 1.11.1.14; diarypropane:oxygen, hydrogen peroxide oxidoreductases, and Laccase (Lac) or E.C. 1.10.3.2; benzenediol:oxygen oxidoreductases, are generally thought to be responsible for the unspecific attack of lignin (37). Both peroxidases are ferric ion containing heme proteins requiring peroxidase (e.g., H_2O_2) for function, and laccase belongs to the copper containing blue oxidases that use molecular oxygen (O_2). The enzymes have the ability in common to catalyze one-electron oxidations resulting in the formation of reactive free radical species inside of the lignin polymer (38) (Figure 2-14). Subsequently, the radicals undergo spontaneous reactions leading to the incorporation of oxygen (O_2) bond cleavages, and finally to breakdown of the lignin molecule (38). LiP and Laccase react directly with aromatic substrates, whereas MnP takes effect via chelated Mn^{3+} ions acting as low-molecular weight redox mediator. Thus, the function of MnP is the generation of Mn^{3+} from Mn^{2+}, which is the actual substrate of the enzyme. Similar to the catalytic cycle of other peroxidases including LiP that of MnP involves the formation of peroxidase compounds II and I. The latter are highly reactive and abstract one electron from Mn^{2+} to form Mn^{3+}. MnP catalysis has an absolute requirement for Mn chelating, organic acids (e.g., oxalate, malate, malonate), which increase the

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affinity of Mn^{2+} to the enzyme and stabilize the extremely reactive Mn^{3+}. Mn^{3+} is small enough to diffuse into the compact lignocellulose complex, where it preferentially reacts with phenolic lignin structures. Therefore, the primary attack on lignin in most fungi is supposedly brought about by the MnP system (37, 39). An exception is the wood degrading fungus *Polyporus cinnabarinus*, which developes an alternative mediator system for the primary attack on lignin, based on laccase and the fungal metabolite 3-hydroxy antheanilic acid (40). The latter is first transformed by laccase into a reactive radical, which then can act in a similar way as chelated Mn^{3+} inside the lignocellulose complex. Another widespread fungal metabolite, veratyl alcohol life is too short to act as an electron mediator (41).

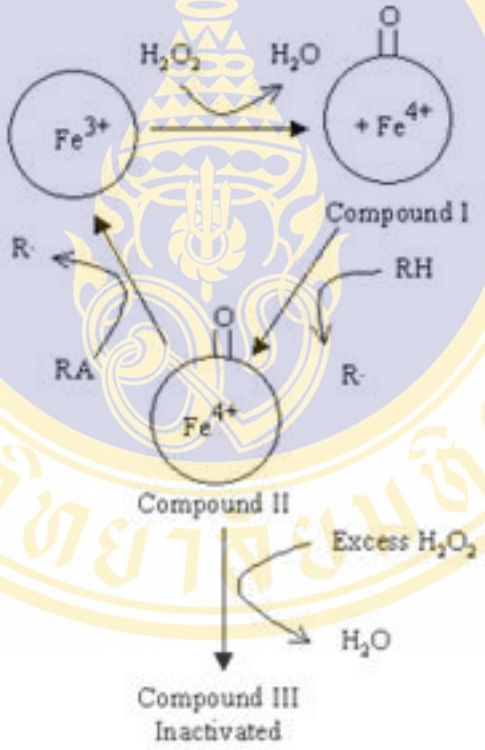

Figure 2-14 The catalytic cycle of LiP (42)

As the result of MnP or laccase catalyzed primary attack, water-soluble lignin fragments are formed that are accessible for the further conversion by ligninolytic enzymes. Laccase oxidizes phenolic structure, whereas LiP preferentially cleaves recalcitrant non-phenolic lignin moieties. MnP is also involved in the further degradation of the formed lignin fragments. Thus, there are indications that the MnP

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system directly mineralizes aromatic lignin structures. It is the only enzyme that partly converts aromatic compounds including lignin to carbon dioxide (CO_2). This means, lignin and other aromatic compounds can be mineralized, at least in part, outside the fungal hyphae (43) (Figure 2-15). The reactions underlying the MnP catalyzed mineralization are still not completely understood, but as much is certain, decarboxylation reactions caused by Mn^{3+} are the final step in this process. Furthermore, certain radicals (carbon-centered radicals, peroxyl radicals, superoxide) deriving from the auto catalytic decomposition of organic acids in the presence of Mn^{3+} may also be involved in the mineralization process (44). The oxidative strength of the MnP system can be considerably enhanced in the presence of additional mediating agents such as unsaturated fatty acids or organic thiols. Both groups of substances are converted by Mn^{3+} to highly reactive radical species (peroxyl and alkoxy radicals of fatty acids, thiol radicals), which increase lignin mineralization (CO_2 formation) and render the cleavage of structures possible that are normally not attacked by the MnP system (e.g., non-phenolic aromatic aryl ethers) (45). The process of MnP catalyzed mineralization of lignin and other substances has been described as enzymatic combustion (46).

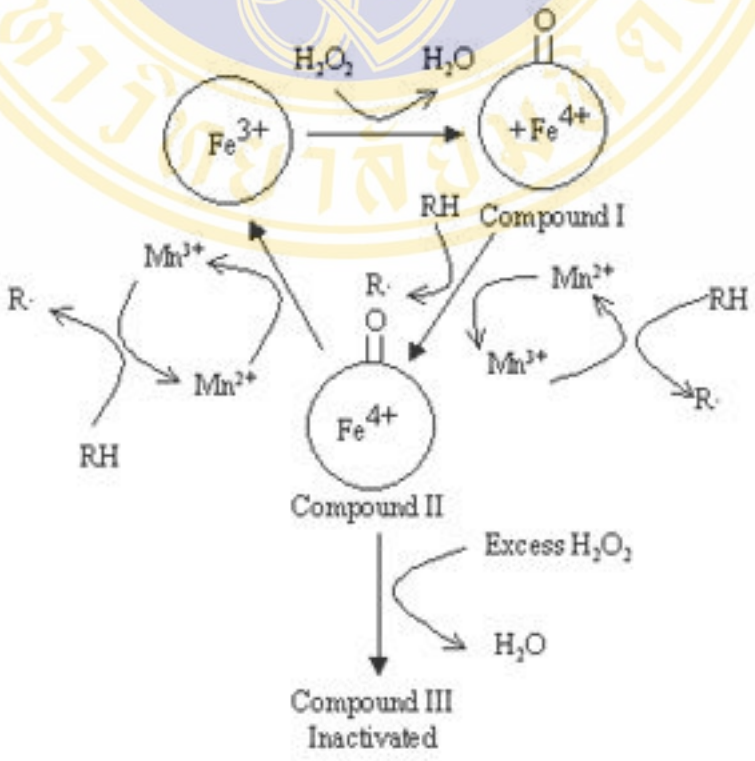

Figure 2-15 The catalytic cycle of MnP (42)

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2.4 Degradation of Aromatic compounds

The unique ligninolytic enzymes system of basidiomycetes based on a highly reactive free radical depolymerization mechanism is ideal for the biodegradation of organopollutants in the environment. Comparing other potential bioremediation systems, the extracellular, non-specific, non-stereo selective lignin-degrading system of basidiomycetes has the advantage of being applicable to a variety of recalcitrant and toxic chemicals. Examples of selected fungi responsible for some degradation were shown in Table 2-1.

Table 2-1 Fungi strains and degradable aromatic chemicals (2)

Fungus	Organopollutants
Bjerkandera adusta	Benzo[*a*]pyrene, other PAHs, TNT, dyes
Nematoloma frowardii	Benzo[*a*]pyrene, other PAHs, TNT, PCP, AsO
Phanerochaete chrysosporium	Benzo[*a*]pyrene, other PAHs, BTX, DNT, TNT, DDT, DCP, PCP, PCBs DCA, dyes, polystyrenes, KCN
Phanerochaete sordida	PAHs, polychlorinated DBDs and DBFs
Phlebia radiata	TNT, dyes
Pleurotus ostreatus	Benzo[*a*]pyrene, other PAHs, dibenzothiophene, TNT
Stropharia rugosoannulata	DCP, PCP, TNT
Trametes versicolor	Benzo[*a*]pyrene, other PAHs, DCA, PCP, dyes

Among these substances are hazardous xenobiotic compounds like polychlorinated di-benzodioxins and di-benzofurans, other chlorinated aromatics, nitro aromatic compounds (explosive), and carcinogenic organopollutants belonging to the polycyclic aromatic hydrocarbons. MnP, LiP, and Laccase have been shown to be involved in the transformation of organopollutants (47). As the result of ligninolytic enzymes attack on organopollutants, various metabolites, which either could be further degraded intracellularly, coupled to the humans or mineralized by MnP system, are

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formed. The latter has been shown to mineralize various organopollutants directly in cell-free system indicating that an extracellular “enzymatic combustion” of hazardous chemical is possible (46). The proposed pathway of an organopollutant degradation is shown in Figure 2-16.

Over the last decade, efforts have been made to use ligninolytic basidiomycetes in bioremediation technologies. Laboratory experiments have shown wood inhabiting white-rot fungi stimulate the degradation of certain organopollutants (i.e., PAHs, PCP, TNT) in contaminated soil. However, a disadvantage of these fungi is their small competitive potential in soil. One of the best-investigated and highly active white-rot fungus ***P. chrysosporium*** can not compete with other microorganisms and survives in soil only for a very short time. Therefore, research has recently been focused on litter-decaying basidiomycetes that naturally colonize the upper soil layers. ***Stropharia rugosoannulata*** is an example of such a fungus that has already been successfully tested for TNT decontamination of soils (48).

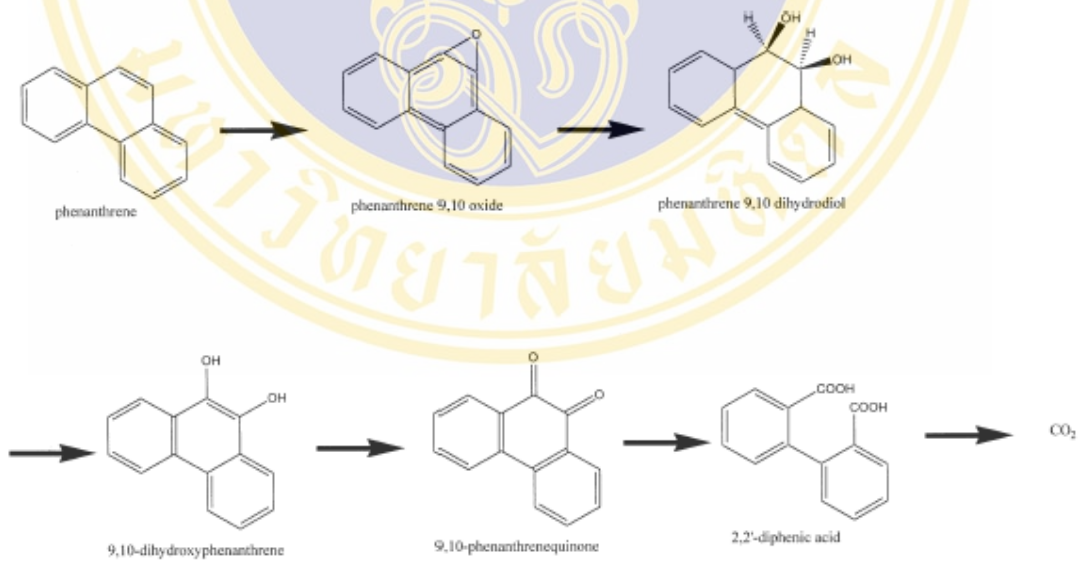

Figure 2-16 Proposed degradation pathway for phenanthrene by white-rot fungi. The formation of dihydrodiol, quinone, and diphenic acid metabolites has been shown to vary among species (2)

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2.5 Application of polymeric dye

In general, selected method for measurement of intermediated or end products of the lignin degradation resulting from fungi are C^{14} labeling method. However, this method is very complicate and time consuming. The development of assays utilizing polymeric dyes as substrates for the lignin degrading system have facilitated these screening procedures. The dyes are inexpensive and can be obtained commercially in high purity. They are stable and readily soluble, have high extinction coeffients, and have low toxicity toward *Phanerochete chrysosporium* and other white-rot fungi and bacteria tested (49). *O*-Anisidine and other low-molecular-weight dyes that have been used in similar assays could be taken into cell, whereas polymeric dyes will remain extracellular, at least during the initial stages of degradation, and thus will provide a better model for lignin degradation system (49). Fungal decolorization of polymeric dyes has led to the development of simple, rapid, and quantitative spectrophotometric assays for the lignin degrading system in microorganisms (49).

Several studies with lignocellulolytic microorganisms demonstrated that the presence of microbial peroxidases seems to be correlated with their abilities to decolorize certain dyes (50, 51, 52, 53, 54). The first dyes to be used as substrates in lignin biodegradation assays were three polymeric dyes, Poly B-411, Poly R-481, and Poly Y-606 (50). Decolorization of Poly B-411 has since been used in several studies to screen microbial cultures for lignocellulose degradation abilities, including fungi (51, 52), bacteria (53), soil microbial populations (54) and genetically transformed strains (55). The dye Remazol Brilliant Blue R (RBBR) has also been used for this purpose in fungi (56) and bacteria (57).

The decolorization of the polymeric dyes Poly R-478 and Poly B-411 by 170 strains of white-, brown-, and soft-rot decay fungi and non wood-decaying xylophilous fungi has been studied (50). Primary objective in these researchs was to relate dye decolorization to known ligninolytic activity and to the presence of phenoloxidases and/or peroxidases. There is no simple relation between the presence of Mn(II)-peroxidase (MnP) or lignin peroxidase (LiP) and the decolorization of the Poly R-478 and Poly B-411.

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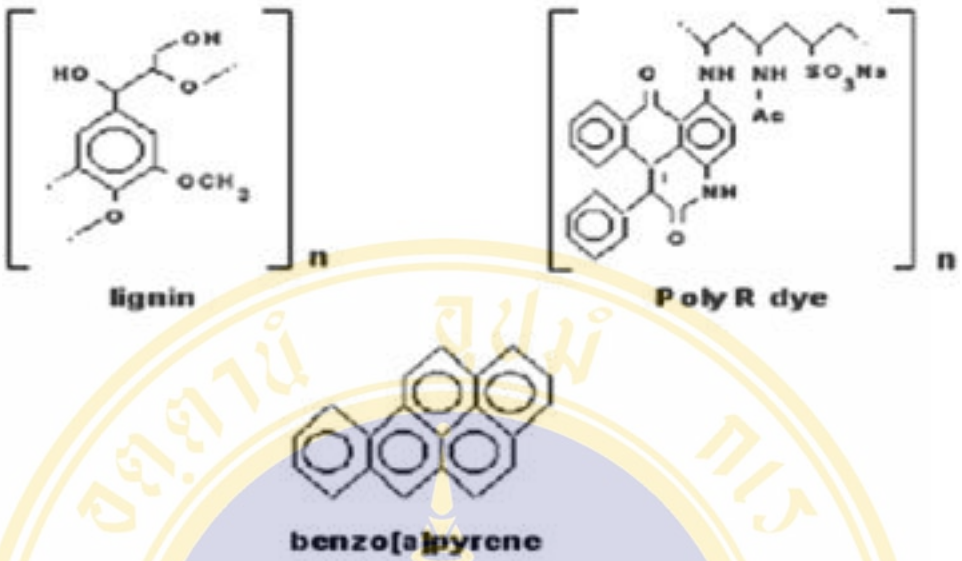

Figure 2-17 Similarity of Lignin, Poly R dye, and Benzo[*a*]pyrene structue (2)

The measurement of decolorization is done by adding 0.02% (w/v) dye to a media culture. Then, decreasing of absorbance values of the dye at specific wavelength (520 nm and 350 nm for Poly R-478) can be observed (7). While, qualitative decolorization on the agar mediums has also been applied by observing clear zone (58).

2.6 Enzymes assay

The quantitative assay of each LMEs catalytic activity is conducted spectrometrically. Due to Beer's law, $A = \hat{I}bc$, where b (length of cell; generally 1 cm) and $\hat{I}$ (molar absorptivity) are held constant. A (absorbance) obtains from a spectrophotometric measurement. While, c (concentration) can be calculated from the formula.

Determination of LiP activity is done in associate to amount of increasing veratraldehyde ($\hat{I}_{310\ nm}$ *ca* 9300 $M^{-1}cm^{-1}$) resulting from oxidization of veratryl alcohol in H_2O_2 added condition (59).

Unlike LiP activity, Lac activity can be measured from varies substrates such as syringaldazine, phenol red, 2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), and 2,6-di-methoxyphenol (DMP) DMP is oxidized to orange/brown dimer ($\hat{I}_{468\ nm}$ *ca* 10000 $M^{-1}cm^{-1}$) (60).

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The H_2O_2-dependent formation of an orange/brown product from DMP is used to assay MnP activity. The reaction is started with addition of H_2O_2. Then, the correction with Lac activity will be performed (7).

2.7 Relevant research

The estimated amount of annually produced TNT (2,4,6-trinitrotoluene) is about 900,000 kg (2). It is well known that under anaerobic conditions certain bacteria carry out reductive transformation of TNT, although mineralization does not occur (2). In addition, a photo catalytic TiO_2-assisted reduction of TNT has been found, but without significant mineralization. Transformations of TNT result in formation of dinitrotoluenes (DNTs) 2-amino-4,6-dinitrotoluene, 2,6-diamino-4-nitrotoluene, and 4-amino-2,6-dinitrotoluene. These compounds are generally not degraded further, or are dimerized to even more persistent azo- and azoxy dimmers (61). TNT and other munitions waste are constant problems for the military. Not only does the detonation risk of explosives render them hazardous, but also the constituent compounds are also toxic and persistent in the environment.

Another research has shown that white-rot fungi are able to transform TNT to DNTs, as are many bacteria and mitosporic fungi. Only white-rot fungus, *P. chrysosporium*, has shown significant capability of degrade DNT and mineralize it to CO_2 (61).

Organochlorine insecticides such as dichlro-diphenyl-tri-chroethane (DDT), lindane, and the aldrins (aldrin, dieldrin, endosulfan) have been produced in vast quantities and wildly used since the 1940s. Organochlorine herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and 2-methyl-4,6-dichlorophenoxyacetic acid (MCPA) have been wildly applied, with the concomitant generation of dioxin. They are persistent in the environment resulting to biomagnification through the food chain (1).

For a DDT degradation study, *P. chrysosporium* plus *Pleurotus ostreatus*, *Phellinus weirii*, and *Polyporus versicolor* have been chosen. The result indicated the possibility of mineralization of DDT between 5.3-13.5% (62). Another study, on degradation of 1,1-dichloro2,2-bis(4-chlorophenyl)ethane (DDE), an extremely toxic and even more persistent metabolites from broken down DDT also showed that DDE

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could be mineralized to $^{14}CO_2$ by *P. chrysosporium* (63). The mineralization of the dioxin 2,7-dichlorodibenzo-*p*-dioxin by *P. chrysosporium* has also been demonstrated (64).

Polychlorinated biphenyls (PCBs) are produced by chlorination of biphenyl. Several different congeners are produced which vary in their degree of substitution. They have a variety of industrial uses such as dielectric fluids, organic diluents, plasticizers and solvent extenders. They have been extensively used in every country of the world (1). Numerous studies have shown that white-rot fungi including *Coriolopsis polyzona*, *P. chrysosporium*, *P. ostreatus*, and *T. versicolor* are capable of significant PCB removal *in vivo* (65, 66, 67).

Synthetic dyes are chemically diverse, with those commonly used in industry divided into those of azo, triphenylmethane or heterocyclic/polymeric structure. They are not commonly biodegradable and many are toxic. (68). They are used extensively in the biomedical, foodstuff, plastic, and textile industries, where 10-14% of dye is lost in effluent during the dying process (69).

White-rot fungi have been shown as superior dye decolorizers, particularly in comparison to prokaryotes which generally poor or non-decolorizers (70). Even the lignin-transforming actinomycetes, *Streptomyces chromofuscus*, is a weak decolorizing microorganism comparing to *P. chrysosporium* (71).

Bleach-plant effluent from chlorine mediated process of high-quality paper production contains usually 5-10% residual lignin. As a result, large aqueous volumes of toxic, low-molecular-mass, halogenated lignin degrading products are released into the environment from bleach-plants. These include chloro-lignins, chloro-phenols, chloro-guaiacols, chloro-cate-chols, and chloro-aliphatics (72).

Oxidative demethylation and dechlorination of bleach-plant effluent, with associated decolorization, has been demonstrated for *P. chrysosporium* (72). In a study by Neilson and et al, seven white-rot fungal species were evaluated for their ability to depolymerize polyvinylchloride (PVC), a widely used synthetic textile.

Plastics are widely used in almost every parts of the world. Their contribution to landfill and high visibility as discarded waste due to their recalcitrance make studies on plastic biodegradability of particular environmental relevance (2).

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P. chrysosporium, T. Versicolor, and Pleurotus sajor caju achieved significant depolymerization with lower levels of depolymerization observed in four other *Pleurotus* species (2).

The organic wood preservatives creosote and pentachlorophenol (PCP) have been wildly used due to their high efficacy, although there has largely been discontinued as a result of environmental concerns. Creosote is a coal-tar distillation product, comprising a highly heterogeneous PAH mixture. PCP is a benzene ring with five Cl-substitutions. PCP is not considered as pollution threat in wood preservation process, but contamination of soil and groundwater at manufacture and treatment plants has raised environmental concerns (2).

Evidence for creosote-mineralizing capability among white-rot fungi can be inferred from PAH-mineralizing abilities. It is important to consider creosote as a complex mixture that may be more toxic to fungi than single PAH or simple mixtures of the component PAH. One study has shown that soft-rot fungi isolated from creosote-treated wood can actively grow on filter paper impregnated with 100% w/w creosote (73). The biodegradation of PCP has been extensively researched. The complete mineralization of PCP, involving oxidation and reductive dehalogenizing steps, has been recorded for *P. chrysosporium* that mineralized up to 50.5% of ^{14}C-radiolabeled PCP (74).

The oxidation of a model PAH compound, anthracene, in the presence of cosolvents by the white-rot fungus, *Bjerkandera* sp. strain BOS55 was investigated by Field and et al (75). When solvents up to 20% (v/v) were added to 9-day-old cultures, ligninolytic activity as indicated by Poly R-478 dye decolorization and anthracene oxidation was evident for several days. Since 20% solvent was toxic to cells, the oxidation of anthracene can be attributed to extracellular peroxidases, which were shown to tolerate the solvent. Additions of 11%–21% (v/v) acetone or ethanol increased the rate of anthracene bioconversion to anthraquinone in liquid medium by a factor of 2–3 compared to fungal cultures receiving 1%–3% solvent.

Veratryl alcohol, which is a normal secondary metabolite of the lignin degradation, was found to enhance the action of LiP on many substrates (76). Moreover, aromatic compounds were found to stimulated growth and enzymes production (77). Lignin degradation is highly oxygen dependent. Higher oxygen

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tension increases peroxidase and 14-ligninolytic activities, as well as the production of H_2O_2 and veratryl alcohol. Medium pH has been found to be critical to lignin degradation with a pH optimum at about 4.5 and repression of degradation below 3.5 and over 5.5 (78).

White-rot fungi can rapidly oxidize high-molecular-weight polycyclic aromatic hydrocarbons (PAH) to polar metabolites, but only limited mineralization takes place. ^{14}C-radiolabeled benzo[*a*]pyrene was subjected to oxidation by the white rot fungus *Bjerkandera* sp. strain BOS55. After 15 days, up to 8.5% of the [^{14}C]benzo[*a*]pyrene was recovered as $^{14}CO_2$ in fungal cultures, up to 73% was recovered as water-soluble metabolites, and only 4% remained soluble in dibutyl ether (79). Thin-layer chromatography analysis revealed that many polar fluorescent metabolites accumulated. Addition of indigenous micro flora to fungal cultures with oxidized benzo[*a*]pyrene on day 15 resulted in an initially rapid increase in the level of $^{14}CO_2$ recovery to a maximal value of 34% by the end of the experiments (>150 days), and the level of water-soluble label decreased to 16% of the initial level. In fungal cultures not inoculated with micro flora, the level of $^{14}CO_2$ recovery increased to 13.5%, while the level of recovery of water-soluble metabolites remained as high as 61%. No large differences in $^{14}CO_2$ production were observed with several inoculates, showing that some polar metabolites of fungal benzo[*a*]pyrene oxidation were readily degraded by indigenous microorganisms, while other metabolites were not (79).

Polypores were isolated from several forests in Lombok Island, Indonesia and screened for their ability to degrade lignin. Sixty of sixty-five samples isolated were tested using a qualitative plate assay through direct visualization of agar plate decolorization containing the polymeric dye Poly R-478 (0.02% $^{w}/_{v}$). Fifteen isolates were able to decolorize the dye, indicating a lignin-degrading ability. Spectrophotometric enzyme assays from all selected isolates were carried out to examine the production of ligninolytic enzymes (laccase, lignin peroxidase and manganese peroxidase). Twelve selected isolates produced all three kinds of enzymes tested, but *Hexagonia tenuis*, *Inonotus patouillardii*, and *Stereum* sp. only produced laccase and lignin peroxidase. The importance of this study to support biotechnology in the paper industry is discussed (58).

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The polymeric dyes Remazol Blue, Poly B and Poly R are clearly not suitable for a fast assay, since their decolorization by the peroxidases was too slow, being detectable only after 4 h. Also, based on the data after 24 hours incubation, Poly B and Remazol Blue did not distinguish between MnP and LiP, and Poly R was hardly oxidized, though it seemed to be specific for LiP. Phenol Red, though reported to be used as a substrate for detecting Mn(II)-peroxidase in the presence of lactate and bulk protein, was not decolorized under our assay conditions by MnP. It was oxidized by LiP (80).

Eight rapid Poly R-478 dye-decolorizing isolates from Netherland were screened in this study for the biodegradation of polycyclic aromatic hydrocarbons (PAHs) supplied at 10 mg liter^{-1}. Several well known ligninolytic culture collection strains, *P. chrysosporium BKM-F-1767*, *T. versicolor Paprican 52*, and *Bjerkandera adusta CBS 595.78* were tested in parallel. All of the strains significantly removed anthracene, and nine of the strains significantly removed benzo[*a*]pyrene beyond the limited losses observed in sterile liquid and $HgCl_2$-poisoned fungus controls. None of the strains accumulated PAH quinones during benzo[*a*]pyrene degradation. Biodegradation of PAH by the various strains was highly correlated to the rate by which they decolorized Poly R-478 dye, demonstrating that ligninolytic indicators are useful in screening for promising PAH-degrading white-rot fungal strains (81).

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CHAPTER III
RESEARCH METHODOLOGY

The purpose of this study is to screen and isolate white-rot fungi with high LMEs activity. Samples of fruiting body of basidiomycetes and pieces of rotten wood were collected from several Thai forests. High potential LMEs producing strains were determined from ability to degrade the dye poly R-478 and subsequent results of LMEs assays. The experimental procedures shown in Figure 3.1 as follows:

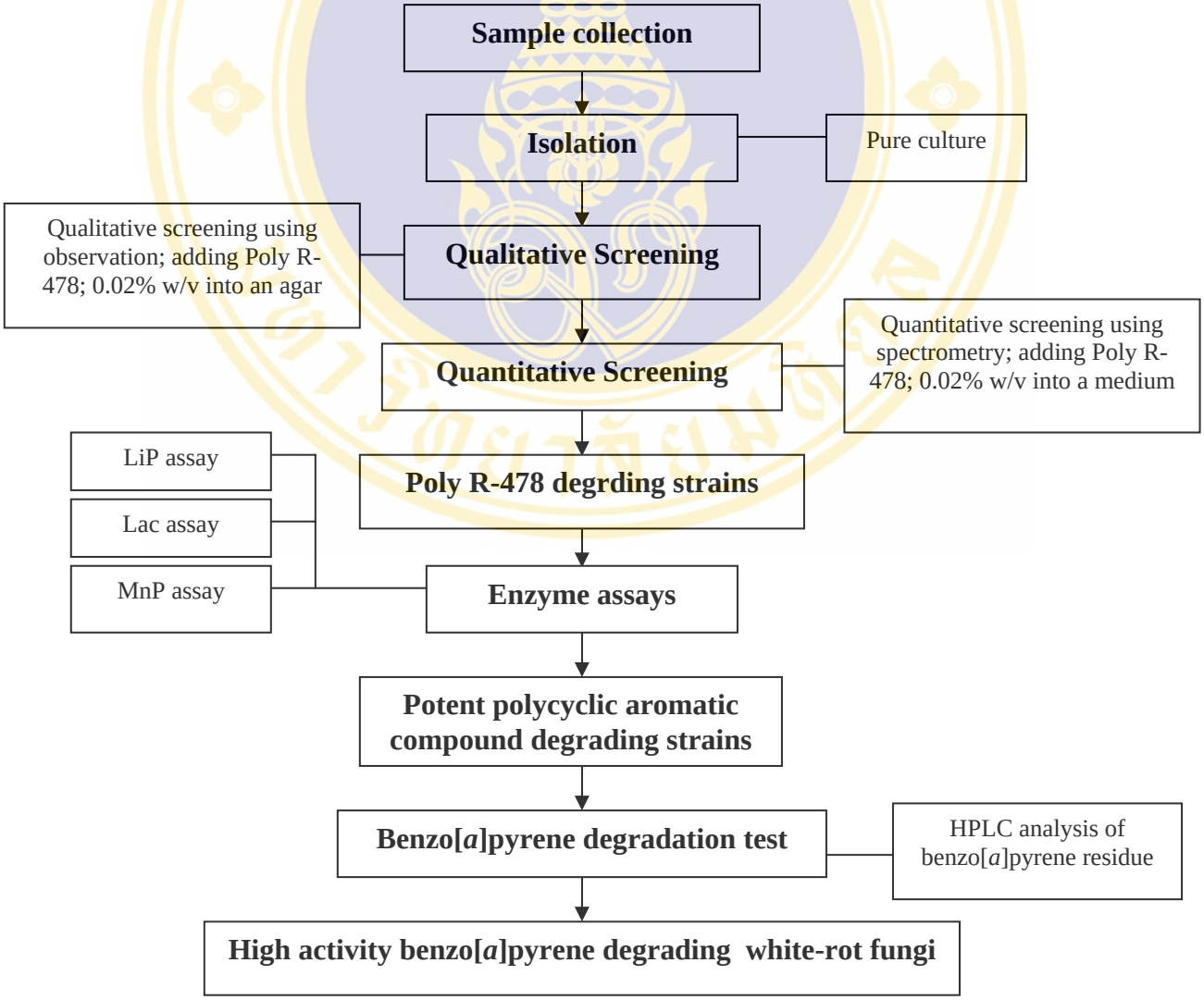

Figure 3-1 Experimental procedures

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3.1 Chemical reagent, instruments, and equipments

3.1.1 Chemical reagents

All Chemical reagents are analytical grade, except chemical reagent numbers 21 to 23 are HPLC grade.

1. Yeast extract
2. Citric acid
3. $(NH_4)_2SO_4$
4. K_2HPO_4
5. $MgSO_4.7H_2O$
6. $CaCl_2.2H_2O$
7. Agar
8. Poly R-478
9. Malt extract
10. Streptomycin sulfate
11. Sodium tartrate
12. Veratryl alcohol
13. H_2O_2
14. 2,6-di-methoxyphenol (DMP)
15. Succinic acid dimethyl ester (Sodium 2,2-dimethylsuccinate) (DMS)
16. Sodium malonate
17. $MnSO_4$
18. Acetone
19. Ethyl acetate
20. DMSO
21. Benzo[*a*]pyrene
22. Methyl alcohol
23. dH_2O (mobile phase)
24. Reference Species: *Trametes versicolor*

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3.1.2 Instruments and Glassware

1. Paper filter, 0.45 μm
2. Cellulose acacetate filter, 0.45 μm
3. pH meter
4. Hot plate
5. Auto pipette
6. Scalpel blade
7. Forceps
8. Plastic bags
9. Spirit lamp
10. Petri dish
11. Erlenmeyer flask
12. Beaker
13. Pipette
14. Cylinder
15. Volumetric flask
16. Blender

3.1.3 Equipment

1. Hot air oven; Conterm digital series
2. Autoclave; Hirayama HV-25/50/85/110
3. Centrifuge with temperature control; Hettich Rotina 35R zentrifugen
4. Spectrophotometer with temperature control; Shimudzu UV-1601
5. HPLC with C-18 column; Walters P5-HPLC

3.1.4 Screening media

The media is modified from Muzariri's media (82): per litre distilled water

1. Yeast extract 5 g
2. $(NH_4)_2SO_4$ 5 g
3. K_2HPO_4 5 g

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4. $MgSO_4.7H_2O$ 0.5 g
5. $CaCl_2.H_2O$ 0.02 g

The mixture was placed in a volumetric flask and adjusted to the 1 litre-mark with distilled water. Then the media was autoclaved at 121°C for 15 minute.

3.2 Methodology

3.2.1 Sample collection

White-rot fungi samples were collected in several forest areas of northern, northeastern, western, southern and central parts of Thailand. Either fresh or dry basidiocarps of the white-rot fungi at the size of 1 x 1 inch were collected from live trees, dead trees, or logs.

3.2.2 Isolation

The isolation was carried out as follows: The collected basidiocarp was cleaned thoroughly with sterile-distilled water prior to the experiment. To cultivate the sample, a spirit lamp was used to sterilize scalpel blades, which were used to open up a clean surface within the basidiocarp and to incise it into small sections. A clean tissue of about 1 x 0.5 cm was lifted for subsequent inoculation on the agar plates. Other samples such as pieces of rotted wood or a small amount of soil were also placed on the center of plates. Each sample was incubated at 32°C for at least 3 days after which fungal growth could be observed. For the Isolation process, A sterile needle was used to transfer each mycelium with different appearance for subsequent inoculation onto malt extract agar plates. Every isolate was purified by successively streaking three times on sterile agar plates. The final isolate was stored on a malt extract agar slant at 4°C.

3.2.3 Qualitative screening by using polymeric dye (58)

The isolated fungus (from 3.2.2) was grown on a media agar containing chemicals (per litre distilled water) as described in 3.1.4 plus 15 g agar. After autoclaving, 0.2 g sterile poly R-478 and 0.5 g sterile streptomycin, using 0.45 μm membrane filter, were added into the media.

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To screen dye degradable fungus strains, each isolate was transferred to cultivate on an agar plate using a 6 mm-plug. Every plate was incubated at 32°C for at least 14 days for later clear zone observations. Only clear zone producing white-rot fungi was selected for later process.

3.2.4 Quantitative dye degrading assay (7)

The selected fungus from 3.2.3 was grown in a media agar containing (per litre distilled water) as described in 3.1.4. After autoclaving, a 6 mm-plug was added into the 250ml-flask containing 50 ml of the media. Then, the cultures were placed in an incubator at 32°C. On 7-day, filtered poly R-478 was added into the culture up to 0.2% (w/v). After the dye has been added, 1 ml of the media broth was drawn every 24 hours for the spectrometric assay until A520/A350 ratio reach 0.8-1.0 or incubation period of 240 hours. Percentage of decolorization was quantified in triplicate by comparing the A520/A350 ratio with that of authentic standards. Before the measurement, the broth was diluted 10 fold with distilled water. Absorbance of the samples at 520 and 350 nm were determined. The fungal strains with similar or significantly higher activity were selected for further assay and cultivated up to 30-day for decolorizing end point.

3.2.5 Enzyme assay

After degradation activity of dye resulting from each isolate was compared with that of *Trametes versicolor*, the strains with similar or significantly higher activities were selected for enzyme assays. For each strain, six 250 ml-flasks with 50 ml medium (3.1.4) were prepared and autoclaved. A 6 mm-plug of white-rot fungi was inoculated in to each flask. The dye poly R-478 was added into three flasks to provide the end concentration of 0.02% (w/v). The cultures were then incubated at 32°C. Every 24 hours, the absorbance A520/A350 ratio of each culture was monitored. When the ratio fell between 1.0 and 0.8 the remaining three flasks were ready for enzyme assays

For enzyme assay, the culture broth was centrifuged at 200g for 30 min to separate mycelium from culture broth. All enzyme assays were done using a spectrophotometer at 30°C. One unit of enzyme was calculated from 1 mM of substrate, which was oxidized by each enzyme.

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For each enzyme, the enzyme unit was determined as follow:

3.2.5.1 Lignin peroxidase (LiP)

The reaction mixture contained up to 550 µl culture broth in 50 mM sodium tartrate with pH2.5 and 2 mM veratryl alcohol in 1 ml solution. The reaction was started with 0.4 mM H_2O_2 and the formation of veratraldehyde ($\varepsilon_{310\ nm}$ *ca* 10000 M^{-1} cm^{-1}) (59) was monitored (7).

3.2.5.2 Laccase (Polyphenol oxidase)

The oxidation of 2,6-dimethoxyphenol (DMP) to an orange/brown dimer was used for measuring laccase activity ($\varepsilon_{468\ nm}$ *ca* 10000 M^{-1} cm^{-1}) (60). The reaction mixture contained up to 750 µl culture broth in 50 mM DMS, pH4.5 and 1mM DMP in a total volume of 1ml.

3.2.5.3 Manganese peroxidase (MnP)

The H_2O_2-dependent formation of an orange/brown product from DMP was used to monitor MnP activity. The reaction mixture contained 50 mM sodium marlonate pH 4.5, 1 mM DMP, 1 mM $MnSO_4$, and up to 650 µl culture broth in a total volume of 1 ml. The reaction was started by the addition of 0.4 mM H_2O_2. Then the result was corrected with laccase activity.

3.2.6 Benzo[*a*]pyrene degradation

For each selected strains from 3.2.5, six 250 ml-flasks with 50 ml medium (3.1.4) were prepared. Before autoclaving, a 0.02-ml aliquot of acetone containing 1 mg of benzo[*a*]pyrene with 50 µl of dimethyl sulfoxide (DMSO) was added to three of the flasks to provide a final benzo[*a*]pyrene concentration of 20 µg l^{-1} in the culture medium. After autoclaving, a 6mm-plug of white-rot fungi was inoculated to each flask. The dye poly R-478 was added into the remained three flasks to provide concentration of 0.02% (w/v) and subsequently incubated at 32°C. The absorbance ratio was monitored every 24 hr until the absorbance ratio of the poly R-478 added flasks fell between 1.0 and 0.8. Then the remaining three benzo[*a*]pyrene added flasks were ready for HPLC analysis as following:

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The entire content of the flask was grounded in a high-speed blender for 30 s. The slurry was filtered through Whatman no. 4 filter paper and extracted twice with 300 ml of ethyl acetate/dH_2O (5:1, v:v). The organic extracts were evaporated to dryness, the residual was resuspended in 10 ml of acetone. The suspension was filtered through a 0.45 μm cellulose acetate filter prior to HPLC determination. The measurement was conducted using a Walter HPLC attached with a C-18 column. A gradient elution was used with an initial concentration of MeOH:H_2O, 100:0 (v:v) at a flow rate of 1 ml/min with detection at 254 nm. Benzo[*a*]pyrene concentrations were quantified by comparison with an authentic standard, and the amount of benzo[*a*]pyrene degraded was calculated.

3.2.7 Data analysis

The values of LMEs activities, %decolorization of poly R-478 and %degradation of benzo[*a*]pyrene were obtained from three individual measurements. The differences of values were compared using analysis of variance (one-way ANOVA). When F-ratios of data are statistically differ at level 0.05, each of the average differences were ranked using Dunnett's test to compare each treatment with reference strain. All of the statistical analysis was performed with a computer package program Prism 2.01 for Windows.

For calculation the values of %decolorization of poly R-478 using formula as following: $(A520/A350)_1 / (A520/A350)_0 \times 100$

While, $(A520/A350)_0$ was the absorbance ratio at 520 and 350 at starting time and $(A520/A350)_1$ was the absorbance ratio at 520 and 350 at ending time of the measurement.

For calculation the values of %degradation of benzo[*a*]pyrene using formula as following: $B[a]P_1 / B[a]P_0 \times 100$

While, $B[a]P_0$ was amount of B[*a*]P residue at starting time and $B[a]P_1$ was amount of B[*a*]P residue at ending time of the measurement.

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CHAPTER IV
RESULTS

4.1 Sample collections

White-rot fungi samples were collected in forest regions of northern, northeastern, western, southern and central Thailand. Either fresh or dry basidiocarps of the white-rot fungi were collected from live trees, dead trees, or logs.

In northern Thailand, the collection was performed at Fang, Chaing Mai province on Doi Pa Hom Pok, which average temperature was below 20°C and humidity was high.

In western Thailand, the collection was performed at Suan Peung, Rachaburi province in arid area. The temperature, during the time of sampling, ranged between 24 and 35°C.

In eastern Thailand, the collection was performed in Trat province at Khao Chamao. The temperatures, during the time of sampling, ranged between 24 and 30°C.

In northeastern Thailand, the collection was performed in Srisaket, Korat and Leoi province. Srisaket site was the driest and hottest site. The temperatures, during the time of sampling, ranged between 25 and 33°C. The sample collections in Korat were performed at Khao Yai. The temperatures, during the time of sampling, ranged between 24 and 32°C. For Leoi, the sample collections were performed at Phu Kradeung. The temperatures, during the time of sampling, ranged between 11 and 32°C.

In southern Thailand, the collection was performed in Prajuabkirikhan and Ranong. In Prajuabkirikhan the sample collections were performed in Khao Samroiyod, which located adjacent to the sea. The temperatures, during the time of sampling, ranged between 25 and 33°C. In Ranong, the collections were performed at Ngaw Waterfall. The temperatures, during the time of sampling, ranged between 24 and 30°C and the humidity was very high.

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4.2 Isolation

The collected samples were isolated using malt extract agar. From Srisaket, Trat, Prajuabkirikhan and Ranong provinces, the numbers of isolated white-rot fungi were less than the other areas. This due to fact that the collections in those regions were performed during the hot and dry period. Therefore, there were almost no white-rot fungi strains that form a basidiocarp were observed. The results were shown in Table 4-1.

Table 4-1 Sampling location and amount of isolate

Province	Location	Code	Isolate
Chaing Mai	Doi Pahom Pok	C	18
Srisaket	Muang Srisaket	S	4
Korat	Khao Yai	K	21
Leoi	Phu Kradeung	P	25
Rachaburi	Suan Peung	R	22
Trad	Khao Chamao	T	8
Prajuabkirikhan	Khao Samroiyod	KS	5
Ranong	Ngaw Waterfall	RN	6
Total			109

4.3 Qualitative screening by using polymeric dye

All of the 109 isolated fungi was cultivated using 0.02% poly R-478 containing media agar to screen out poly R-478 decolorizing fungi. The dye decolorization was determined by clear zone observation. The Table 4-2 showed that, only 26 isolates had dye-decolorizing activities.

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Table 4-2 Number, percentage of dye-decolorizing strains and relative codes

Location	Number of dye-decolorizing strain	%dye-decolorizing strains	Strain Code
Doi Pahom Pok	6	33.33	C2, C4, C5, C6, C11, C16
Muang Srisaket	2	50.00	S2, S4
Khao Yai	6	28.57	K4, K8, K10, K14, K17, K18
Phu Kradeung	2	8.00	P2, P5
Suan Peung	6	27.27	R1, R4, R6, R7, R8, R9
Khao Shamao	1	12.50	T5
Khao Samroiyod	2	40.00	KS1, KS4
Ngaw Waterfall	1	16.67	RN5
Total	26	23.85	

4.4 Quantitative dye-decolorizing assay

All of 26 isolates that showed dye decolorizing activity were cultivated in media broth for 7 days, then poly R-478 was added. The culture serum was drawn for measuring every 24 hours after poly R-478 had been added until the A520/A350 ratio reach 0.8-1.0 or incubation period of 240 hours. The dye-decolorizing activity was calculated from decolorizing slope, 10^3(A520/A350) Hr^{-1}, and the strains that have significantly higher activity than that of the reference strain, *Trametes versicolor*, were further cultivated up to 30-day to obtain the end value, 10^3(A520/A350).

From the 6 dye-decolorizing strains collected from Doi Pahom Pok, only C6 had the dye-decolorizing activity higher than that of the reference strain, *T. versicolor*. It reduced the A520/A350 ratio down to below 1.0 within 168 hours. The results were shown in Figure 4-1.

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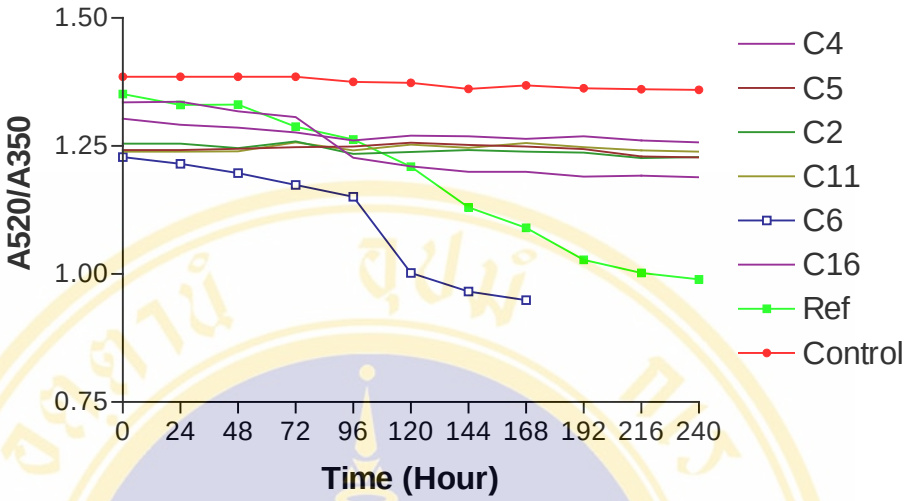

Figure 4-1 Dye-decolorizing activity of fungi collected from Doi Pahom Pok under laboratory condition at 32^{O}C comparing with that of *Trametes versicolor*

Figure 4-2 showed the comparison of dye-decolorizing activities of the two fungal strains collected from Srisaket comparing to that of the *T. versicolor*. Only the strain S4 had higher decolorizing rate than that of the reference strain. It reduced the A520/A350 ratio down to below 1.0 within 192 hours.

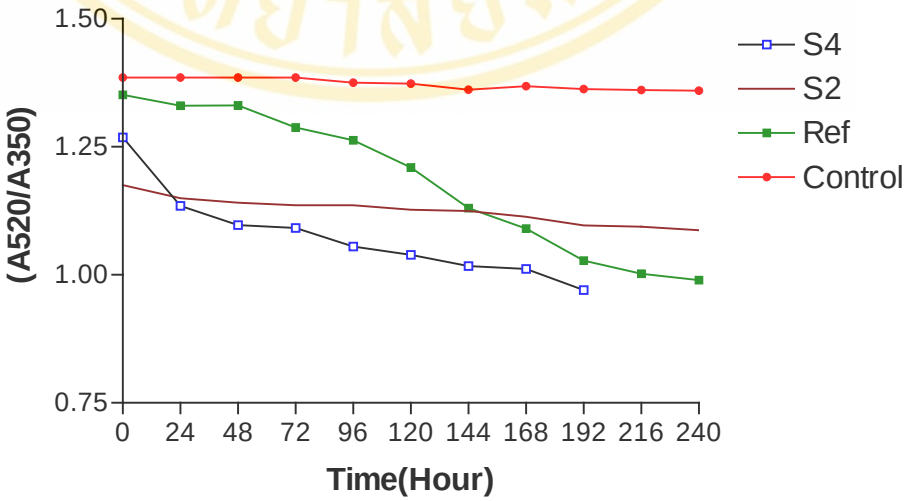

Figure 4-2 Dye-decolorizing activity of fungi collected from Srisaket under laboratory condition at 32^{O}C comparing with that of *Trametes versicolor*

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From the 6 dye-decolorizing strains collected from Khao Yai, only K8, K10, K14 and K18 showed higher dye-decolorizing rates than that of the reference strain, *T. versicolor*. They reduced the A520/A350 ratio down to below 1.0 within 168, 168, 216 and 216 hours, respectively. The results were shown in Figure 4-3.

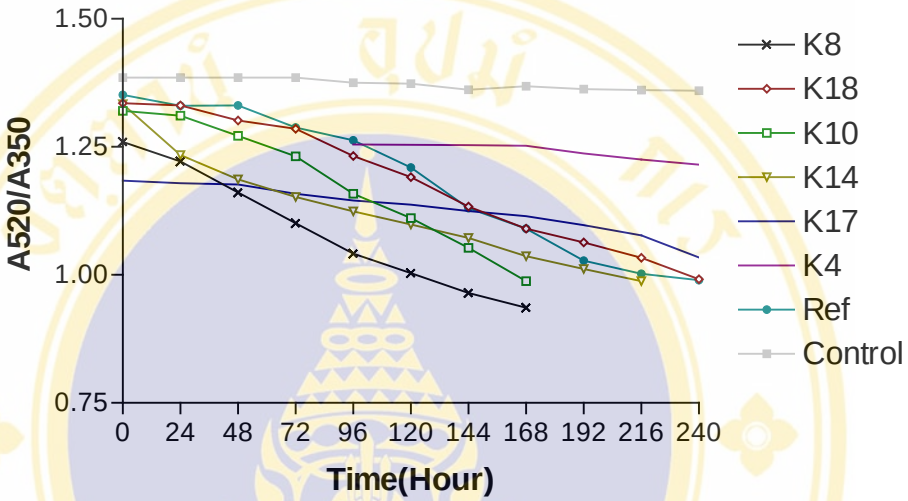

Figure 4-3 Dye-decolorizing rate of fungi collected from Khao Yai under laboratory condition at 32^{O}C comparing with that of *Trametes versicolor*

Figure 4-4 showed the comparison of dye-decolorizing rate of the two fungal strains collected from Phu Kradeung and that of the *T. versicolor*. Only P5 had higher decolorizing rate than that of the reference strain. It reduced the A520/A350 ratio down to below 1.0 within 216 hours.

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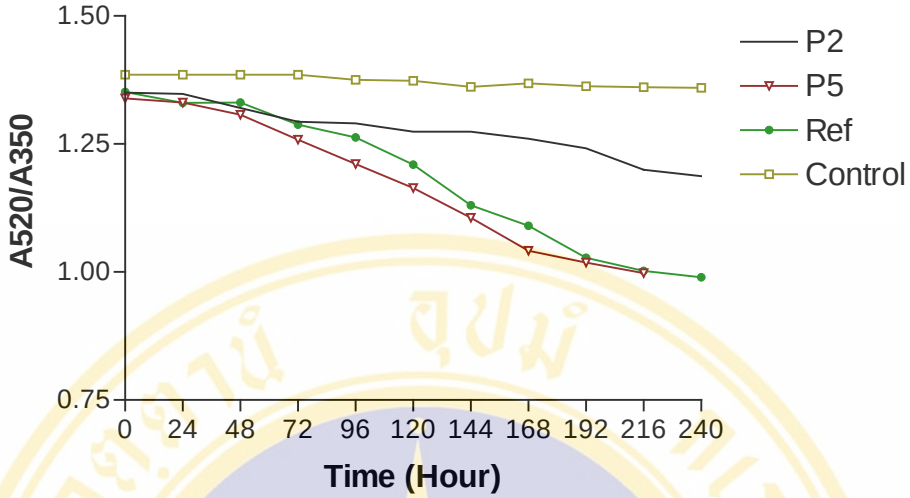

Figure 4-4 Dye-decolorizing rate of fungi collected from Phu Kradeung under laboratory condition at 32^{O}C comparing with that of *Trametes versicolor*

From the 6 dye-decolorizing strains collected from Suan Peung, only R6 and R7 had the dye-decolorizing rate more than that of the reference strain, *T. versicolor*. They reduced the A520/A350 ratio down to below 1.0 within 192 hours. The results were shown in Figure 4-1.

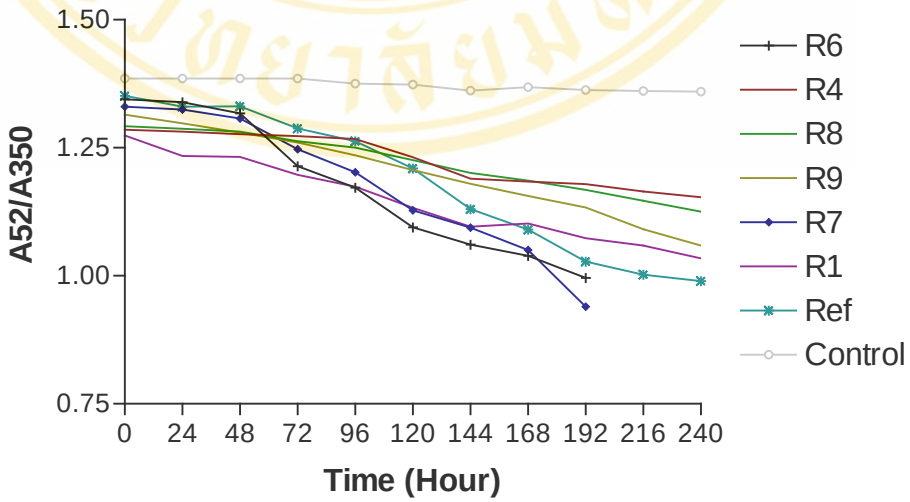

Figure 4-5 Dye-decolorizing rate of fungi collected from Suan Peung under laboratory condition at 32^{O}C comparing with that of *Trametes versicolor*

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Figure 4-6 showed the comparison of dye-decolorizing rate of T3 stain collected from Khao Chamao comparing to that of *T. versicolor*. It reduced the A520/A350 ratio down below 1.0 within 216 hours.

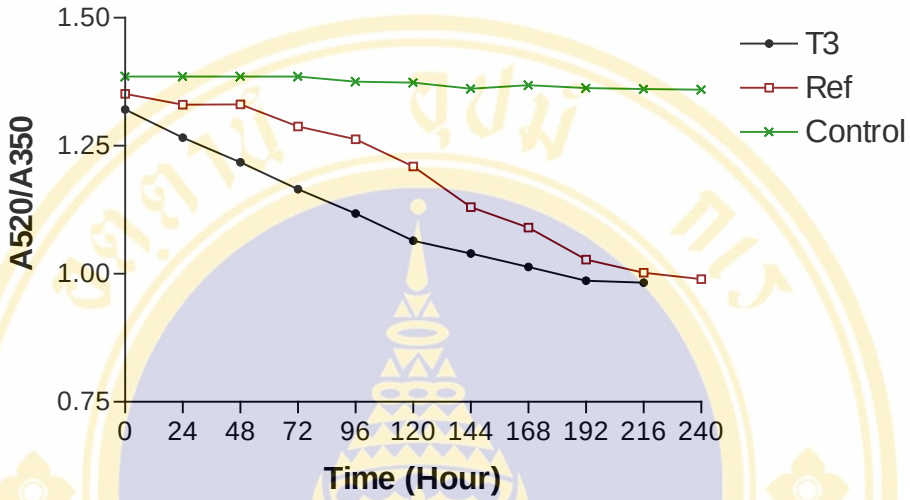

Figure 4-6 Dye-decolorizing rate of fungi collected from Khao Chamao under laboratory condition at 32^{O}C comparing with that of *Trametes versicolor*

Figure 4-7 showed the comparison of dye-decolorizing rate of the two fungi strains collected from Khao Samroiyod and that of the *T. versicolor*. There was not a single strain that had higher dye-decolorizing rate than that of the reference strain, *T. versicolor*.

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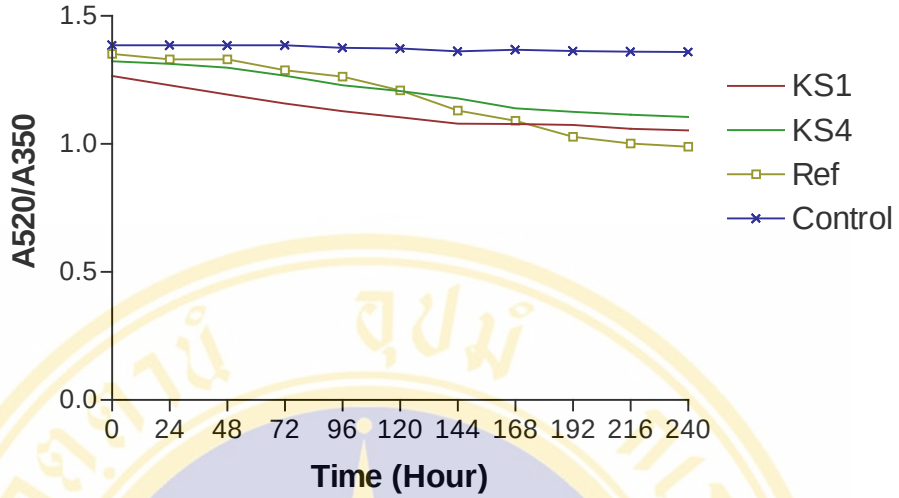

Figure 4-7 Dye-decolorizing rate of fungi collected from Khao Samroiyod under laboratory condition at 32^{O}C comparing with that of *Trametes versicolor*

For samples from Ngaw Waterfall, no strain that had dye-decolorizing rate higher than that of *T. versicolor* was observed after 240 hours incubation period.

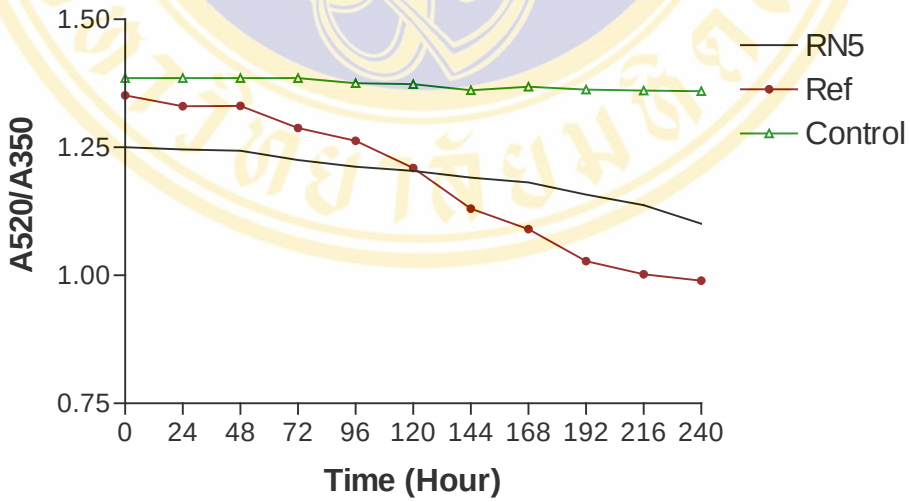

Figure 4-8 Dye-decolorizing rate of fungi collected from Ngaw Waterfall under laboratory condition at 32^{O}C comparing with that of *Trametes versicolor*

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Figure 4-9 showed poly R-478 decolorizing activity of all the fungal strains expressing as absorbance ratio decreasing rate ($10^3(\Delta A520/A350)/h^{-1}$) comparing to the activity of the reference strain, *T. versicolor*, which was 1.50, 1.98 decolorizing activity at $32^{O}C$ of the strain K10 was considered the highest.

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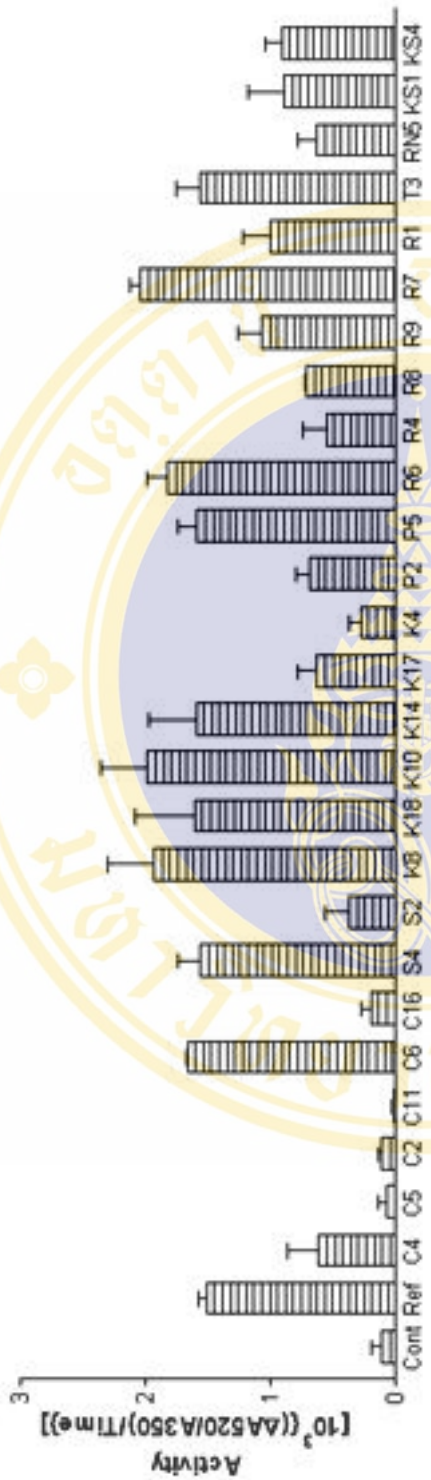

Figure 4-9 Dye decolorizing activity of isolated white-rot fungi strains, comparing with that of the reference strain, *T. versicolor*, at 32^{O}C after 240 hours incubation period

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In order to classify fungal strains in the groups according to their decolorizing activities, Duncan's statistic were performed on the results. The strains, which had equal or significant higher dye-decolorizing activity than *T. versicolor*, were selected for further enzymes and benzo[*a*]pyrene degradation assay. The results were shown in Table 4-3.

Table 4-3 Results of post hoc test on dye-decolorizing activity of different fungal strains, using Duncan's statistic

DYE

	STRAIN	N	Subset for alpha = .05										
			1	2	3	4	5	6	7	8	9	10	11
Duncan[a]	C11	3	.011389										
	C5	3	.063056										
	C2	3	.109167										
	C16	3	.193097	.193097									
	K4	3	.274768	.274768	.274768								
	S2	3	.369361	.369361	.369361	.369361							
	R4	3		.549292	.549292	.549292	.549292						
	C4	3			.610000	.610000	.610000	.610000					
	K17	3			.623611	.623611	.623611	.623611					
	RN5	3			.623611	.623611	.623611	.623611					
	P2	3				.679583	.679583	.679583	.679583				
	R8	3				.707222	.707222	.707222	.707222				
	KS1	3					.885139	.885139	.885139				
	KS4	3					.905556	.905556	.905556				
	R1	3						.999583	.999583				
	R9	3							1.063889				
	ref	3								1.507875			
	s4	3								1.552995	1.552995		
	t3	3								1.565278	1.565278		
	p5	3								1.580093	1.580093		
	k14	3								1.592100	1.592100	1.592100	
	k18	3								1.602997	1.602997	1.602997	
	c6	3								1.660516	1.660516	1.660516	1.660516
	r6	3								1.815452	1.815452	1.815452	1.815452
	k8	3									1.927381	1.927381	1.927381
	k10	3										1.978571	1.978571
	r7	3											2.032639
	Sig.		.071	.062	.079	.093	.080	.055	.053	.131	.066	.051	.056

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

The results from Duncan's statistic in Table 4-3 classified the observed strains into activity group levels. The strains with decolorizing activity as high as that of the reference strain, *T. versicolor*, were S4, T3, P5, K14, K18, C6, and R6. While only three strains, including K8, K10 and R7 were found to have significantly higer activity. Therefore, 10 strains were selected for further experiments and the incubation period was extended to 30-day to determine the end point activity values, which use to determine the trend of decolorizing potential for long period assay. The results showed that a steady decolorizing rate within 30 days was produced by the strains K8 and K4.

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Table 4-4 End value of poly R-478 decolorization after 30-day incubation in absorbance ratio (10^3(A520/A350) of selected fungi

Code	End value	%Decolorization
Control	1330.6	3.94
Ref	1005.87	25.56
R7	966.85	27.80
R6	956.83	28.84
C6	844.21	31.27
K10	809.00	38.69
K14	836.57	33.85
K18	890.07	33.34
K8	652.83	48.13
S4	470.37	58.29
P5	804.86	39.87
T3	852.42	35.45

4.5 Enzyme assay

Three further enzyme assays, including lignin peroxidase, laccase and manganese peroxidase, were performed on the select fungal strains resulted from 4.4. The results were as follows:

4.5.1 Lignin peroxidase assay (LiP)

The lignin peroxidase activity was determined from the formation of veratraldehyde, which was the product from the reaction between LiP and veratryl alcohol. The Figure 4-10 showed the LiP activity of the observed strains, which ranged between 0.00 nmol min^{-1} (ml sup)$^{-1}$ for R7 and 6.84 nmol min^{-1} (ml sup)$^{-1}$ for K18.

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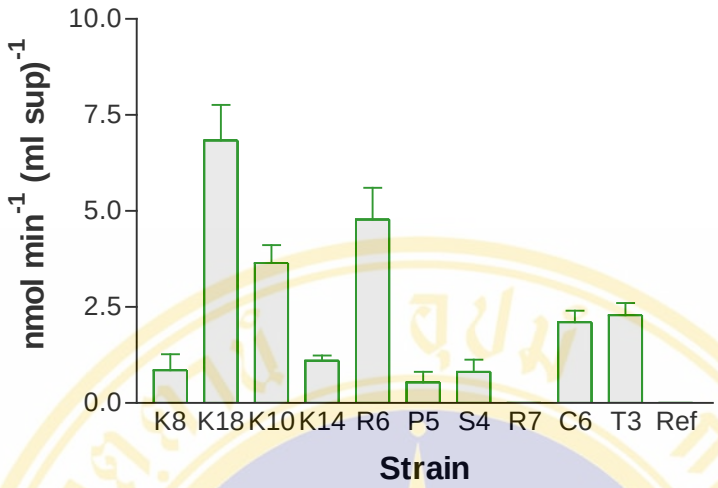

Figure 4-10 Lignin peroxidase activity

Dunnett's statistic was performed to compare the LiP activity of the observed strains with the reference strain's activity. The results were shown in Table 4-5.

Table 4-5 Comparison of different white-rot fungi – Lignin peroxidase (LiP) activity, using Dunnett's statistic

Multiple Comparisons

Dependent Variable: LIP

	(I) STRAIN	(J) STRAIN	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Dunnett t (2-sided)[a]	r7	ref	.000000	.6492927	1.000	-1.920064	1.920064
	r6	ref	4.778500*	.6492927	.000	2.858436	6.698564
	c6	ref	2.100100*	.6492927	.027	.180036	4.020164
	k10	ref	3.643033*	.6492927	.000	1.722969	5.563097
	k14	ref	1.100023	.6492927	.480	-.820041	3.020087
	k18	ref	6.836567*	.6492927	.000	4.916503	8.756631
	k8	ref	.851609	.6492927	.742	-1.068455	2.771673
	s4	ref	.816153	.6492927	.778	-1.103911	2.736217
	p5	ref	.538170	.6492927	.970	-1.381894	2.458234
	t3	ref	2.290467*	.6492927	.014	.370403	4.210531

*. The mean difference is significant at the .05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

From statistical comparison result, the LiP activity of R6, C6, K10, K18 and T3 were significantly higher than that of *T. versicolor*.

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4.5.2 Laccase assay(Polyphenol oxidase)

The laccase activity was determined from the oxidation of 2,6-dimethoxyphenol (DMP) to an orange/brown dimer (60). The Figure 4-11 showed that the laccase activity of the observed strains ranging between 0.00 nmol min^{-1} $(ml\ sup)^{-1}$ for K8 and 1.45 nmol min^{-1} $(ml\ sup)^{-1}$ for K10.

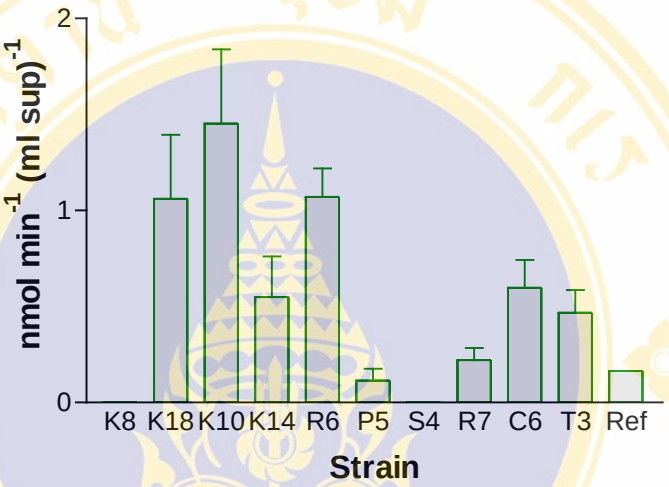

Figure 4-11 Laccase activity of 10 different white-rot fungi comparing with that of the reference, *T. versicolor*

Dunnett's statistic was performed to compare the laccase activity of the observed strains with the reference strain's activity. The results show in Table 4-6.

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Table 4-6 Comparison of different white-rot fungi – Laccase (Lac) activity, using Dunnett's statistic

Multiple Comparisons

Dependent Variable: LAC

Dunnett t (2-sided) [a]

(I) STRAIN	(J) STRAIN	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
r7	ref	.055500	.2595963	1.000	-.712169	.823169
r6	ref	.905767*	.2595963	.016	.138098	1.673435
c6	ref	.433233	.2595963	.497	-.334435	1.200902
k10	ref	1.287800*	.2595963	.000	.520131	2.055469
k14	ref	.383833	.2595963	.626	-.383835	1.151502
k18	ref	.895967*	.2595963	.017	.128298	1.663635
k8	ref	-.164500	.2595963	.995	-.932169	.603169
s4	ref	-.164500	.2595963	.995	-.932169	.603169
p5	ref	-.050917	.2595963	1.000	-.818585	.716752
t3	ref	.302167	.2595963	.835	-.465502	1.069835

*. The mean difference is significant at the .05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

It was shown that the Lac activities of R6, K10 and K18 were significantly higher than that of the *T. versicolor*.

4.5.3 Manganese peroxidase assay (MnP)

The MnP activity was determined from the formation of an orange/brown product from DMP within H_2O_2-dependent condition (60). The MnP activity was calculated from subtraction with laccase activity. The Figure 4-12 showed that the MnP activity ranging between 0.07 nmol min^{-1} (ml sup)$^{-1}$ for R6 and1.02 nmol min^{-1} (ml sup)$^{-1}$for K10.

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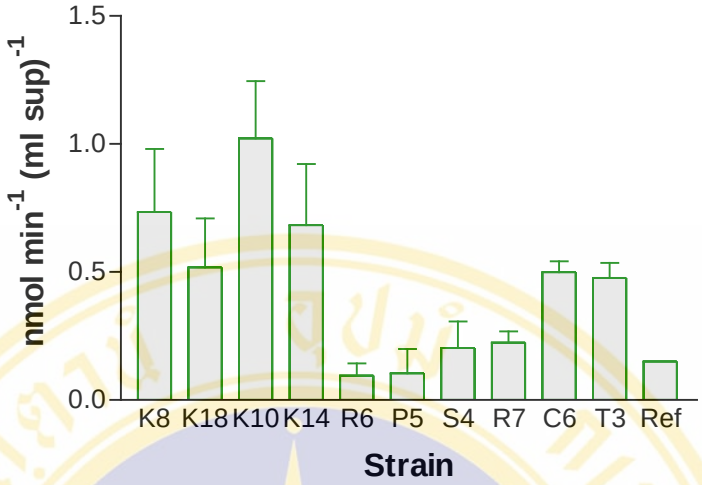

Figure 4-12 Manganese peroxidase activity of 10 different white-rot fungi comparing with that of the reference, *T. versicolor*

Dunnett's statistic was performed to compare the MnP activity of different fungal strains to the reference strain's activity. The results were shown in Table 4-7.

Table 4-7 Comparison of different white-rot fungi – Manganese peroxidase (LMn) activity, using Dunnett's statistic

Multiple Comparisons

Dependent Variable: MNP
Dunnett t (2-sided) [a]

(I) STRAIN	(J) STRAIN	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
r7	ref	.074667	.2065030	1.000	-.535996	.685330
r6	ref	-.054850	.2065030	1.000	-.665513	.555813
c6	ref	.349333	.2065030	.482	-.261330	.959996
k10	ref	.871567*	.2065030	.003	.260904	1.482230
k14	ref	.532990	.2065030	.108	-.077673	1.143653
k18	ref	.367983	.2065030	.425	-.242680	.978646
k8	ref	.583863	.2065030	.066	-.026800	1.194526
s4	ref	.052688	.2065030	1.000	-.557975	.663351
p5	ref	-.045768	.2065030	1.000	-.656431	.564895
t3	ref	.327300	.2065030	.553	-.283363	.937963

*. The mean difference is significant at the .05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

The results showed that only the MnP activitiy of K10 was MnP was significantly higher than that of *T. versicolor*.

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4.6 Benzo[*a*]pyrene degradation

The selected fungal strains from 4.4 were also tested for their benzo[*a*]pyrene degradation abillity. The start benzo[*a*]pyrene concentration was 20 µg l^{-1}. The results were expressed in benzo[*a*]pyrene degrading percentage of the selected strains. The results of %degradation ranged between 8.62 for P5 and 39.85 for K18 as showed in Figure 4-12.

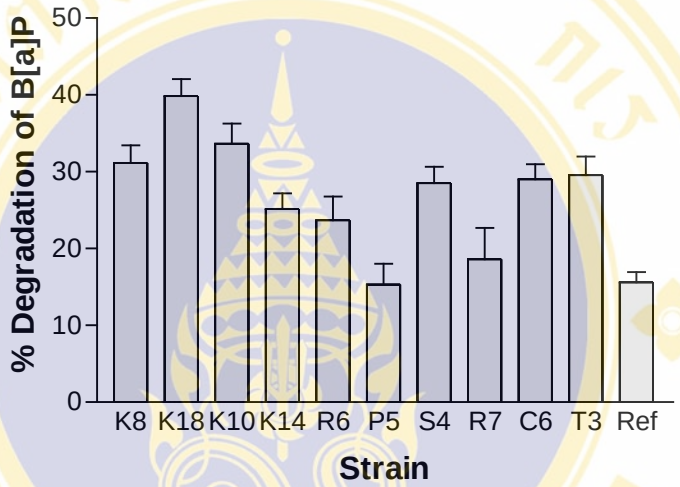

Figure 4-13 Percent degradation of benzo[*a*]pyrene by 10 different strains comparing to the reference strain, *T. versicolor*, under laboratory condition

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Dunnett's statistic was used to compare the B[*a*]P degrading percentage of selected fungi with the reference's activity. The results show in Table 4-8.

Table 4-8 Comparison of Percentage benzo[*a*]pyrene degrading ability of 10 different selected white-rot fungi and the reference, *T. versicolor,* using Dunnett's statistic

Multiple Comparisons

Dependent Variable: BAP

Dunnett t (2-sided) [a]

(I) STRAIN	(J) STRAIN	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
r7	ref	3.000900	3.584315	.968	-7.598504	13.600304
r6	ref	8.062800	3.584315	.200	-2.536604	18.662204
c6	ref	13.391200*	3.584315	.009	2.791796	23.990604
k10	ref	18.042800*	3.584315	.000	7.443396	28.642204
k14	ref	9.516400	3.584315	.093	-1.083004	20.115804
k18	ref	24.235000*	3.584315	.000	13.635596	34.834404
k8	ref	15.552600*	3.584315	.002	4.953196	26.152004
s4	ref	12.905700*	3.584315	.012	2.306296	23.505104
p5	ref	-.283100	3.584315	1.000	-10.882504	10.316304
t3	ref	13.950000*	3.584315	.006	3.350596	24.549404

*. The mean difference is significant at the .05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

The results showed that under laboratory condition at 32^{O}C. Only the strains C6, K10, K18, K8, S4 and T3 had higher ability to degrade benzo[*a*]pyrene than that of the *T. versicolor*.

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CHAPTER V
DISCUSSION

Collection of fungal samples at different time of the year and topographically different area provided for a wider biodiversity of the gene pool of LMEs producing white-rot fungi. In the sites with high humidity and low temperature such as Chaing Mai, most of the samples were basidiocarps and showed clearly high activity in wood degradation. The samples from low humidity area showed less acivity in degrading wood. During the collection period, almost all of them didn't form a basidicarp. Thus, pieces of woods showing different decaying staged were collected instead.

In qualitative screening for LME producing fungi, agar plates method with Poly-478 added were performed. Using this method, the selection of potent LME producing strains easily performed. Indication of them would be the presence of clear zone on the agar plate. Colonies associating to such evidence were then collected. The other dyes used for such screening were Poly B-411 and Poly Y-606, since they could be oxidized by microorganisms at slower rate than poly R-478 (50), they were not the choice for the present study.

The clear zone producing white-rot fungi strains were selected for quantitative screening. The dye, poly R-478, also was utilized. However, many isolated strains from low temperature area, which showed high activity in the sites, had low decolorizing activities than they were expected. This was possibly due to unsuitable incubating temperature at 32°C chosen for this study, which was too high for those strains. Many fungal strains, which were isolated from hot area with low humidity, such as from Rachaburi, also showed lower decolorizing activities than they were expected. Perhaps liquid media was not really the best choice for cultivating purpose of these strains with could withstand long draught in their natural habitats. The experiment temperature at 32°C seemed to be more suitable for the strains from areas with high temperature and humidity, such as Khao Yai, which showed higher activity than strains from others area.

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Several studies revealed other factors, which influence the decolorizing activity, such as nitrogen limitation (61), amount of aromatic compound in the medium (67), unsaturated fatty acids and organic thiols (45). In this study, the screening method revealed several white-rot fungi with significant higher LMEs activities and B[*a*]P degradation than that of the well-known reference strains, *T. versicolor*, as showed in Table 5-1. The primary enzyme responsible for attack wood-lignin was MnP, which should found in every strain of white-rot fungi (2). The MnP was found in all selected strains.

Table 5-1 Comparison of dye decolorizing activity, enzyme activities and ability to degrade benzo[*a*]pyrene of ten different white-rot fungi and the reference *T. versicolor*

Selected Strains	Dye-decolorizing activity (10^3(ΔA520/A350)/h^{-1})	LiP activity (mmol^{-1} min^{-1} ml^{-1})	Laccase activity (mmol^{-1} min^{-1} ml^{-1})	MnP activity (mmol^{-1} min^{-1} ml^{-1})	%degradation of B[*a*]P
K8	1.8154	0.8516	0.000	0.7339	31.16 %
K10	1.9786	3.6430	1.4523	1.0216	33.65 %
K14	1.5921	1.1000	0.5483	0.6830	25.13 %
K18	1.6030	6.8366	1.0605	0.5180	39.85 %
R6	1.8155	4.7785	1.0703	0.0952	23.67 %
R7	2.0326	0.0000	0.2200	0.2247	18.61 %
P5	1.5801	0.5382	0.1136	0.1042	15.33 %
S4	1.5530	0.8162	0.0000	0.2027	28.52 %
C6	1.6605	2.1001	0.5977	0.4993	29.00 %
T3	1.5653	2.2905	0.4667	0.4773	29.56 %
Ref.	1.5079	0.0000	0.1645	0.15002	15.61 %

The correlation statistics in Table 5-2 confirmed the finding by Zeddel (65) and Vaidya (69) that MnP was possibly the primary factor responsible for dye-decolorization level, while laccase was the second most important factor to dye-

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decolorization level. For B[*a*]P degradation the results confirmed Yatome (70) that LiP, Laccase and MnP are primary factors responsible for level of B[*a*]P degradation with high level of confidence (99%) and correlation at 0.685, 0.538 and 0.678, respectively.

Table 5-2 Correlations statistic of all factors in the experiments, including dye decolorizing level, B[a]P degrading percentage, as well as LiP, Lac and MnP enzyme activities

	Dye	B[*a*]P	LiP	Lac	MnP
Dye	1	0.319	0.180	0.410*	0.585**
B[*a*]P	0.319	1	0.685**	0.538**	0.678**
LiP	0.180	0.865**	1	0.768**	0.302
Lac	0.410*	0.538**	0.768**	1	0.516**
MnP	0.585**	0.678**	0.302	0.516**	1

*. Correlation is significant at the 0.05 level (2-tailed)

**. Correlation is significant at the 0.01 level (2-tailed)

The advantage of using polymeric dye, poly R-478, to screen potent LMEs producing white-rot fungi was its simplicity. But, the white-rot fungi with high activity in dye decolorization could resulted with low activity in B[*a*]P degradation. This could be explained by the fact that dye decolorization could be started with only 2 enzymes, laccase and manganese peroxidase, while all LMEs were required for B[*a*]P degradation (2). Therefore, the results from polymeric dye assay could not accurately predicted PAHs degradation rate (49, 79). It was only guidance for the selection of potent PAHs degrading white-rot fungi strains.

In this study, 6 selected white-rot fungi strains, included C6, K10, K18, K8, S4 and T3, showed significant higher benzo[*a*]pyrene degrading activities than that of the reference strain, *T. versicolor*. The strain K18 was found to have the highest benzo[*a*]pyrene degrading ability (Table 5-3).

The results from Duncan's statistic in Table 5-3 showed 5 groups, which had different B[*a*]P degrading activity. The group with the highest activity was group 5 and the lowest activity group was group1. The most active strain was K18, which was significantly different (level of confidence at 95 %) from the rest of the observed strains, except K10.

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Table 5-3 Duncan's statistic of B[*a*]P degrading activity

BAP

Duncan[a]

STRAIN	N	Subset for alpha = .05				
		1	2	3	4	5
p5	3	15.32900				
ref	3	15.61210				
r7	3	18.61300	18.61300			
r6	3		23.67490	23.67490		
k14	3		25.12850	25.12850		
s4	3			28.51780	28.51780	
c6	3			29.00330	29.00330	
t3	3			29.56210	29.56210	
k8	3			31.16470	31.16470	
k10	3				33.65490	33.65490
k18	3					39.84710
Sig.		.397	.098	.076	.212	.098

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

K18 has been identified later as *Phanerochaete* sp. Although, some earlier studies suggested that degrading activities of other white-rot fungi including *Trametes versicolor*, *Pleurotus ostreatus* and *Bjerkandera* sp. might be more effective than *Phanerochaete.* sp. in removing benzo[*a*]pyrene from nitrogen limited liquid media (84). However, the MM media that was used in this study contained nitrogen compound in the form of yeast extract. This could be the reason why the strain K18, *Phanerochaete* sp., showed significant higher degrading ability than the reference strain in this study. Bumpus and Aust (62), reported that the activity of *Phanerochaete chrysosporium* could be enhanced by a high level of biomass.

Comparing the results of enzyme assay and B[*a*]P degradation assay, some isolated white-rot fungi with low LMEs production, such as S4, could provide higher activity in benzo[*a*]pyrene degradation than the reference strain. This might due to the ability of white-rot fungi that could produce several units of LMEs during secondary metabolism (59). Moreover, the LMEs in some fungi are strongly associated with the cell wall, which resulted in low volume of enzymes detected in the culture broth. In addition, there was also possibility that LiP was bound to intracellular or cell wall (7).

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CHAPTER VI
CONCLUSION AND RECOMMENDATION

6.1 Conclusion

This study was concerned with white-rot fungi isolation and screening process. The fungal collections were done in Northern Thailand at Doi Pahom Pok, Northeastern Thailand at Muang Srisaket, Khao Yai and Phu Kradeung, Western Thailand at Suan Peung, Eastern Thailand at Khao Shamao and Southern Thailand at Khao Samroiyod and Ngaw Waterfall. Many samples were collected from basidiocarp, dead and live tree that had been attacked by white-rot fungi. Over 109 strains of white-rot fungi, which 18 strains came from Northern Thailand, 18 strains came from Northeastern Thailand, 22 strains came from Western Thailand, 8 strains came from Eastern Thailand and 11 strains came from Southern Thailand, were isolated.

Each strain was cultured in poly R-478 added agar plates for qualitative screening to find out LMEs producing white-rot fungi. Only 26 strains of 109 isolated strains or 23.85% were screened out for quantitative assay. In quantitative assay, each strain was cultured in poly R-478 added liquid medium to compare decolorizing activity with a reference strain, *T. versicolor*. Only 10 of 26 strains or 38.46%, which decolorizing activity of 7 strains were level and 3 strains were higher compared with the reference, were selected for enzyme assay and benzo[*a*]pyrene degradation.

In enzyme assay, the selected strains were cultured in liquid medium to measure activity of LiP, Lac and MnP. Each enzyme activity of selected strains was compared with *T. versicolor*. In LiP, there were 5 strains (R6, C6, K10, K18 and T3) that showed significantly higher activity than the reference. In Lac, there were 3 strains (R6, K10 and K18) that showed significantly higher activity than the reference. In Lac, there was only K10 that showed significantly higher activity than the reference. In benzo[*a*]pyrene degradation, there were 6 strains (C6, K10, K18, K8, S4 and T3) that showed significantly higher activity than the reference. K18, which

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showed highest activity at 39.85%degradation of benzo[*a*]pyrene, was identified as *Phanerochaete* sp.

6.2 Recommendation

1. To obtain the highest activity of each strain, factors such as temperature or nutrient dietary should be determined.
2. The high molecular weight PAH such as B[*a*]P was too toxic for many microorganisms, many selected strains should provide higher activity in lesser molecular weight PAHs.

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APPENDIX

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Table A-1 A520/A350 absorbance ratio in 240 hours of the fungi group C

Strain	C2			C4			C5			C6			C11			C16		
Hour	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3
0	1.2591	1.2523	1.2525	1.3347	1.3352	1.3356	1.2350	1.2460	1.2465	1.2263	1.2245	1.2342	1.2368	1.2440	1.2355	1.3124	1.2945	1.3030
24	1.2591	1.2523	1.2525	1.3330	1.3364	1.3403	1.2350	1.2460	1.2465	1.2157	1.2046	1.2259	1.2368	1.2440	1.2355	1.3012	1.2811	1.2925
48	1.2476	1.2462	1.2443	1.3106	1.3194	1.3240	1.2426	1.2404	1.2512	1.1755	1.1947	1.2221	1.2386	1.2402	1.2410	1.2987	1.2687	1.2910
72	1.2474	1.2514	1.2783	1.2995	1.3048	1.3149	1.2436	1.2561	1.2438	1.1626	1.1688	1.1910	1.2424	1.2495	1.2797	1.2879	1.2561	1.2864
96	1.2390	1.2400	1.2243	1.0771	1.2891	1.3151	1.2417	1.2530	1.2527	1.0954	1.1552	1.2031	1.2380	1.2454	1.2418	1.2665	1.2457	1.2697
120	1.2420	1.2423	1.2307	1.0328	1.2831	1.3152	1.2490	1.2627	1.2584	1.0012	1.0246	0.9806	1.2515	1.2542	1.2525	1.2618	1.2794	1.2697
144	1.2423	1.2539	1.2312	1.0227	1.2699	1.3058	1.2503	1.2540	1.2525	0.9612	0.9541	0.9830	1.2446	1.2472	1.2470	1.2615	1.2773	1.2687
168	1.2468	1.2408	1.2301	0.9988	1.2887	1.3110	1.2400	1.2502	1.2561	0.9500	0.9441	0.9540	1.2562	1.2547	1.2573	1.2614	1.2754	1.2561
192	1.2417	1.2399	1.2294	0.9914	1.2402	1.3384	1.2360	1.2500	1.2460				1.2447	1.2469	1.2511	1.2570	1.2714	1.2780
216	1.2409	1.2290	1.2100	0.9910	1.2908	1.2942	1.2270	1.2490	1.2134				1.2431	1.2400	1.2409	1.2566	1.2740	1.2524
240	1.2398	1.2247	1.2208	0.9700	1.2700	1.3270	1.2251	1.2480	1.2110				1.2411	1.2358	1.2399	1.2510	1.2689	1.2510

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Table A-2 A520/A350 absorbance ratio in 240 hours of the fungi group K

Strain	K4			K8			K10			K14			K17			K18		
Hour	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3
0	-	-	-	1.2994	1.2073	1.2729	1.3404	1.3384	1.2821	1.3322	1.3109	1.3564	1.1960	1.1554	1.2003	1.3277	1.3392	1.3394
24	-	-	-	1.2556	1.2028	1.2070	1.3459	1.3252	1.2618	1.3136	1.0500	1.3388	1.1920	1.1554	1.1901	1.3276	1.3328	1.3318
48	-	-	-	1.1893	1.1274	1.1661	1.2998	1.2914	1.2228	1.2769	0.9879	1.2948	1.1911	1.1498	1.1888	1.2989	1.3033	1.3030
72	-	-	-	1.1540	1.0722	1.0764	1.2567	1.2529	1.1847	1.2565	0.9428	1.2566	1.1701	1.1195	1.1865	1.2636	1.3026	1.2894
96	1.2543	1.2553	1.2548	1.1194	0.9799	1.0247	1.2016	1.1865	1.0876	1.2301	0.9078	1.2334	1.1592	1.1031	1.1743	1.1647	1.3009	1.2306
120	1.2538	1.2540	1.2539	1.0980	0.9075	1.0044	1.1491	1.1482	1.0350	1.1897	0.8935	1.2112	1.1549	1.0915	1.1661	1.0897	1.3009	1.1816
144	1.2530	1.2538	1.2534	1.0632	0.8432	0.9884	1.1086	1.0720	0.9782	1.1515	0.8738	1.1919	1.1404	1.0999	1.1329	1.0108	1.2618	1.1273
168	1.2519	1.2530	1.2523	1.0307	0.8143	0.9632	1.0740	0.9972	0.8925	1.1200	0.8651	1.1245	1.1196	1.0914	1.1329	0.9764	1.2175	1.0768
192	1.2340	1.2400	1.2371							1.0894	0.8494	1.0958	1.1090	1.0641	1.1184	0.9669	1.1806	1.0437
216	1.2210	1.2300	1.2244							1.0500	0.8364	1.0773	1.1090	1.0490	1.0729	0.9318	1.1284	1.0405
240	1.2000	1.2290	1.2167										1.0721	1.0239	1.0067	0.9080	1.0813	0.9846

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Table A-3 A520/A350 absorbance ratio in 240 hours of the fungi group KS

Strain	KS1			KS4		
Hour	Y1	Y2	Y3	Y1	Y2	Y3
0	1.3280	1.2700	1.2003	1.2800	1.3300	1.3580
24	1.3000	1.2100	1.1765	1.2647	1.3241	1.3497
48	1.2678	1.1876	1.1247	1.2200	1.3310	1.3420
72	1.2361	1.1569	1.0799	1.1980	1.2900	1.3140
96	1.2060	1.1231	1.0536	1.1700	1.2413	1.2756
120	1.1870	1.0915	1.0340	1.1560	1.2200	1.2456
144	1.1510	1.0600	1.0280	1.1370	1.1987	1.2000
168	1.1130	1.0470	1.0740	1.1300	1.1450	1.1450
192	1.0982	1.0360	1.0890	1.1170	1.1365	1.1240
216	1.0724	1.0340	1.0700	1.1000	1.1240	1.1190
240	1.0639	1.0300	1.0671	1.0970	1.1100	1.1090

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Table A-4 A520/A350 absorbance ratio in 240 hours of the fungi group P

Strain	P2			P5		
Hour	Y1	Y2	Y3	Y1	Y2	Y3
0	1.3408	1.3554	1.3541	1.3080	1.3500	1.3590
24	1.3410	1.3490	1.3538	1.3000	1.3421	1.3500
48	1.3389	1.2547	1.3674	1.2800	1.3200	1.3210
72	1.3000	1.2258	1.3552	1.2247	1.2745	1.2769
96	1.2874	1.2243	1.3583	1.1845	1.2236	1.2249
120	1.2800	1.2210	1.3213	1.1200	1.1843	1.1870
144	1.2780	1.2180	1.3260	1.0841	1.1000	1.1340
168	1.2610	1.1970	1.3226	1.0263	1.0400	1.0590
192	1.2448	1.1801	1.2993	1.0100	1.0320	1.0140
216	1.2100	1.1840	1.2060	1.0047	1.0014	0.9870
240	1.2089	1.1797	1.1724			

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Table A-5 A520/A350 absorbance ratio in 240 hours of the fungi group R

Strain	R1			R4			R6			R7			R8			R9		
Hour	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3
0	1.2803	1.2500	1.2910	1.2800	1.2770	1.2987	1.3403	1.2803	1.2803	1.3203	1.3500	1.3200	1.3100	1.2890	1.2780	1.3400	1.3120	1.2920
24	1.2500	1.2290	1.2240	1.2810	1.2703	1.2940	1.3330	1.3421	1.3500	1.3200	1.3381	1.3157	1.3020	1.2800	1.2800	1.3200	1.3000	1.2740
48	1.2236	1.2867	1.1855	1.2701	1.2685	1.2910	1.3067	1.3200	1.3210	1.3067	1.3106	1.3041	1.3010	1.2764	1.2680	1.2976	1.2860	1.2547
72	1.2000	1.2460	1.1456	1.2657	1.2670	1.2850	1.1643	1.2745	1.2769	1.2643	1.2371	1.2408	1.2580	1.2700	1.2600	1.2666	1.2740	1.2396
96	1.1837	1.2390	1.1000	1.2600	1.2610	1.2810	1.1088	1.2236	1.2249	1.2088	1.1927	1.2049	1.2400	1.2644	1.2460	1.2410	1.2640	1.2000
120	1.1420	1.1900	1.0670	1.2240	1.2310	1.2400	1.0582	1.1843	1.1870	1.1582	1.0747	1.1511	1.2363	1.2439	1.1978	1.2350	1.2340	1.1500
144	1.1070	1.1560	1.0246	1.1800	1.1890	1.1994	0.9972	1.1000	1.1340	1.0972	1.0735	1.1121	1.2019	1.2240	1.1765	1.2000	1.2100	1.1287
168	1.0900	1.1310	1.0863	1.1780	1.1825	1.1920	0.9741	1.0400	1.0590	1.0500	1.0300	1.0700	1.1940	1.2047	1.1591	1.1860	1.1860	1.0965
192	1.0780	1.1007	1.0419	1.1700	1.1810	1.1860	0.9608	1.0320	1.0140	0.9120	0.9704	0.9371	1.1720	1.1940	1.1371	1.1700	1.1540	1.0756
216	1.0674	1.0861	1.0234	1.1650	1.1809	1.1490							1.1500	1.1700	1.1190	1.1220	1.1100	1.0400
240	1.0412	1.0623	0.9981	1.1651	1.1799	1.1152							1.1420	1.1230	1.1100	1.1000	1.0940	0.9840

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Table A-6 A520/A350 absorbance ratio in 240 hours of the fungi group RN

Strain	RN5		
Hour	Y1	Y2	Y3
0	1.2960	1.2554	1.2003
24	1.2920	1.2554	1.1901
48	1.2911	1.2498	1.1888
72	1.2701	1.2195	1.1865
96	1.2592	1.2031	1.1743
120	1.2549	1.1915	1.1661
144	1.2404	1.1999	1.1329
168	1.2196	1.1914	1.1329
192	1.1900	1.1641	1.1184
216	1.1900	1.1490	1.0729
240	1.1721	1.1239	1.0067

Table A-7 A520/A350 absorbance ratio in 240 hours of the fungi group S

Strain	S2			S4		
Hour	Y1	Y2	Y3	Y1	Y2	Y3
0	1.1647	1.1365	1.2256	1.3331	1.1192	1.3523
24	1.1541	1.1215	1.1744	1.2331	1.0192	1.1523
48	1.1486	1.1200	1.1551	1.2262	0.9525	1.1135
72	1.1355	1.1191	1.1529	1.2294	0.9616	1.0829
96	1.1355	1.1191	1.1529	1.2136	0.8916	1.0604
120	1.1311	1.1092	1.1407	1.2245	0.8545	1.0382
144	1.1300	1.1070	1.1367	1.2208	0.8400	0.9916
168	1.1100	1.0945	1.1357	1.2347	0.8100	0.9898
192	1.1071	1.0714	1.1114	1.2112	0.7630	0.9368
216	1.1084	1.0720	1.1020			
240	1.1058	1.0701	1.0850			

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Table A-8 A520/A350 absorbance ratio in 240 hours of the fungi group T

Strain	**T3**		
Hour	**Y1**	**Y2**	**Y3**
0	1.3000	1.3090	1.3540
24	1.2600	1.2500	1.2880
48	1.2169	1.1879	1.2480
72	1.1565	1.1428	1.1966
96	1.1301	1.0907	1.1334
120	1.0897	1.0535	1.0500
144	1.0515	1.0380	1.0300
168	1.0200	0.9961	1.0245
192	1.0094	0.9400	1.0095
216	1.0050	0.9364	1.0073

Table A-9 Enzyme activities of selected fungi

Enzyme	lac			MnP			LiP		
Strain	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3
K8	0.0000	0.0000	0.0000	0.3984	0.5871	1.2161	0.0532	1.4726	1.0290
K18	1.2305	0.1175	1.5334	0.0000	0.8890	0.0000	11.6920	2.8387	5.9790
K10	2.2218	1.1231	0.0000	0.0000	0.0000	1.4677	1.9694	2.7145	6.2452
K14	0.2585	0.0000	0.9635	0.9786	0.0210	0.8607	1.2952	1.1710	0.8339
R6	0.7755	1.1898	1.2455	0.1471	0.0000	0.1384	3.9032	4.0097	6.4226
P5	0.0000	0.2115	0.1293	0.2936	0.0191	0.0000	0.0000	0.8694	0.7452
S4	0.0000	0.0000	0.0000	0.3984	0.1677	0.0419	0.1952	1.0468	1.2065
R7	0.1200	0.3403	0.2000	0.1401	0.2800	0.2542	0.0000	0.0000	0.0000
C6	0.6122	0.3401	0.8412	0.4201	0.5640	0.5147	1.8470	1.7548	2.6985
T3	0.4500	0.2701	0.6802	0.3870	0.5877	0.4579	2.4281	2.7580	1.6854
Ref	0.1645	0.1645	0.1645	0.1500	0.1500	0.1500	0.0000	0.0000	0.0000

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Figure A-1 HPLC chromatogram of B[*a*]P, concentration at 20 μg l^{-1}, retention time 9.2 min

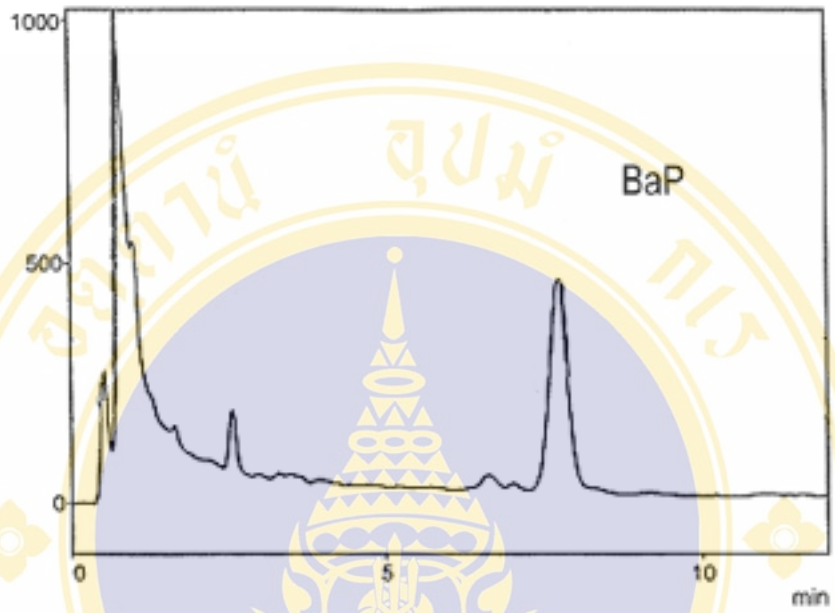

Table A-10 %degradation of B[*a*]P

	%degradation				
Strain	Y1	Y2	Y3	mean	SD
K8	27.54	30.58	35.37	31.16	3.95
K18	43.23	40.66	35.66	39.85	3.85
K10	37.87	28.88	34.21	33.65	4.52
K14	26.72	21.02	27.64	25.13	3.59
R6	17.86	28.48	24.69	23.68	5.38
P5	13.25	12.10	20.64	15.33	4.63
S4	32.44	25.06	28.05	28.52	3.71
R7	25.26	11.23	19.35	18.61	7.04
C6	30.97	31.00	25.04	29.00	3.43
T3	33.20	25.04	30.45	29.56	4.15
Ref	18.07	15.31	13.46	15.61	2.32

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Table A-11 One-way ANOVA of dye-decolorizing activity of all 26 isolated strains

ANOVA

DYE

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	32.962	26	1.268	28.736	.000
Within Groups	2.382	54	.044		
Total	35.344	80			

The result from Table B-1 had proved at least one pair was significant different (Sig. = 0.00 < .05). Than, the post hoc test was performed.

Table A-12 One-way ANOVA of LiP activity of the selected strains comparing with *T. versicolor*

ANOVA

LIP

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	142.472	10	14.247	22.530	.000
Within Groups	13.912	22	.632		
Total	156.384	32			

The result from Table B-2 had proved at least one pair was significant different (Sig. = 0.00 < .05). Than, the post hoc test was performed.

Table A-13 One-way ANOVA of Laccase activity of selected strains and *T. versicolor*

ANOVA

LAC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.188	10	.719	7.111	.000
Within Groups	2.224	22	.101		
Total	9.412	32			

The result from Table B-3 had proved at least one pair was significant different (Sig. = 0.00 < .05). Than, the post hoc test was performed.

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Table A-14 One-way ANOVA of MnP activity of the selected strains and *T. versicolor*

ANOVA

MNP

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.735	10	.273	4.275	.002
Within Groups	1.407	22	.064		
Total	4.142	32			

The result from Table B-4 had proved at least one pair was significant different (Sig. = 0.00 < .05). Than, the post hoc test was performed.

Table A-15 One-way ANOVA of percentage benzo[*a*]pyrene degradation of selected strains and *T. versicolor*

ANOVA

BAP

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1758.120	10	175.812	9.123	.000
Within Groups	423.961	22	19.271		
Total	2182.081	32			

The result from Table B-5 had proved at least one pair was significant different (Sig. = 0.00 < .05). Than, the post hoc test was performed.

Table A-16 Correlations statistic of all factors in the experiments

Correlations

		DYE	BAP	LIP	LAC	MNP
DYE	Pearson Correlation	1	.319	.180	.410*	.585**
	Sig. (2-tailed)	.	.070	.316	.018	.000
	Sum of Squares and Cross-products	2.661	24.329	3.674	2.054	1.942
	Covariance	.083	.760	.115	.064	.061
	N	33	33	33	33	33
BAP	Pearson Correlation	.319	1	.685**	.538**	.678**
	Sig. (2-tailed)	.070	.	.000	.001	.000
	Sum of Squares and Cross-products	24.329	2182.081	399.895	77.031	64.475
	Covariance	.760	68.190	12.497	2.407	2.015
	N	33	33	33	33	33
LIP	Pearson Correlation	.180	.685**	1	.768**	.302
	Sig. (2-tailed)	.316	.000	.	.000	.088
	Sum of Squares and Cross-products	3.674	399.895	156.384	29.448	7.685
	Covariance	.115	12.497	4.887	.920	.240
	N	33	33	33	33	33
LAC	Pearson Correlation	.410*	.538**	.768**	1	.516**
	Sig. (2-tailed)	.018	.001	.000	.	.002
	Sum of Squares and Cross-products	2.054	77.031	29.448	9.412	3.223
	Covariance	.064	2.407	.920	.294	.101
	N	33	33	33	33	33
MNP	Pearson Correlation	.585**	.678**	.302	.516**	1
	Sig. (2-tailed)	.000	.000	.088	.002	.
	Sum of Squares and Cross-products	1.942	64.475	7.685	3.223	4.142
	Covariance	.061	2.015	.240	.101	.129
	N	33	33	33	33	33

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

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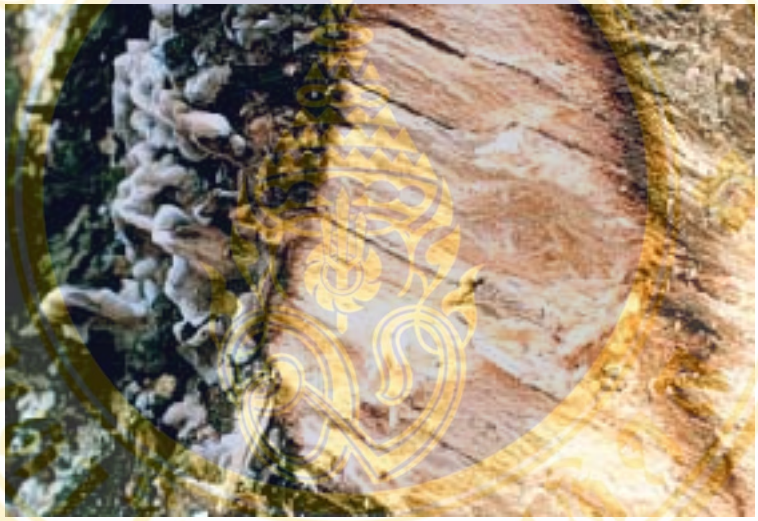

Figure A-2 Bleached wood has been attacked by white-rot fungi

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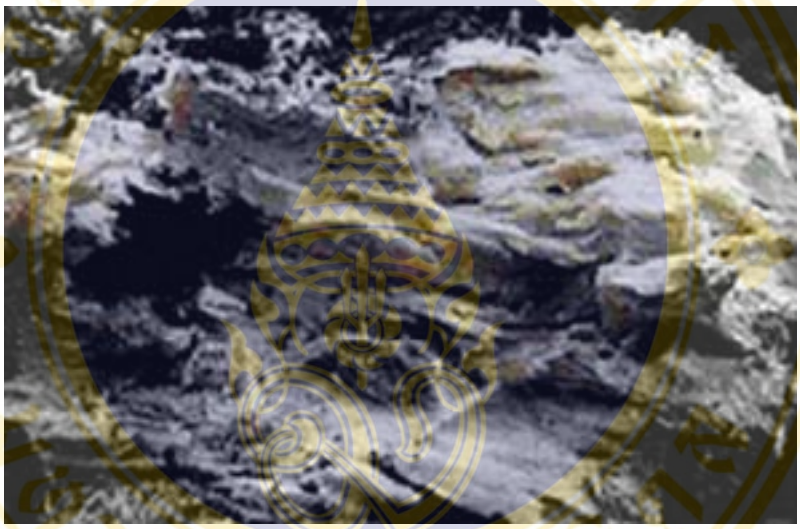

Figure A-3 A piece of rotten wood, which K18 has been isolated

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Figure A-4 Decolorizing activity of white-rot fungi at 0, 7, and 14 day

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Figure A-5 Photo of K18, *Phanerochaete* sp.

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BIOGRAPHY

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