CHAPTER 2

LITERATURE REVIEW

2.1 Environmental problems from dyestuffs

Textile processes consume large amount of water and generated large amount of wastewater. This wastewater is contaminated with organic substances and other chemicals. Dyes are the one of those chemicals usually uses in dying processes. Over 10,000 dyes, with an annual production of over 0.7 million metric ton worldwide, are commercially available and about 5-20% of the dyestuffs are lost in the industrial effluents (Campos *et al.*, 2001; Soares *et al.*, 2001; Young and Yu, 1997) causing the problems as following (Banat *et al.*, 1996):

1. Generate a high visibility to waterbodies. The presence of only small amounts of some dyes in water about 10-15 ppm is highly visible (Campos *et al.* 2001) and causing a visual pollution.
2. Dyes are potentially converted to toxic and carcinogenic substances. Most of dyes used are synthetic dyes produced from known toxic and carcinogenic precursors such as benzidine (Figure 2.1) and other aromatic compounds. Under anaerobic condition such as in river sediments, the dyes can be converted to aromatic amines by reduction reaction of some anaerobic microorganisms which pose a more serious biotoxic threat than the origin (Novotny *et al.*, 2001). In recent years, there are more than 20 kinds of aromatic amines that can be potentially reduced and released from dyestuffs (Table 2.1; Eskilsson *et al.*, 2002; Reife *et al.*, 1999).
3. Reduce water transparency by their high visibility. This problem affect directly on photosynthetic organisms in aquatic ecosystems such as phytoplanktons and aquatic plants.
4. Competition to oxygen solubility when dissolved in large amount in water leading to the decreasing of dissolved oxygen, which directly affect on aquatic lives.

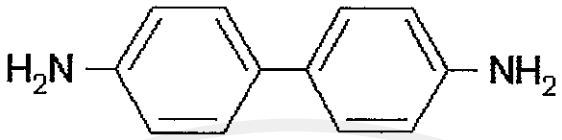


Figure 2.1 Structure of benzidine, one member of aromatic amines

Table 2.1 List of specified toxic and carcinogenic aromatic amines

o-Aminoazotoluene
4-Aminobiphenyl
Benzidine
p-Chloroaniline
4-Chloro-*o*-toluidine
3,3'-Dichlorobenzidine
3,3'-Dimethoxybenzidine
3,3'-Dimethylbenzidine
4-Methoxy-*m*-phenylenediamine
6-Methoxy-*m*-toluidine
4,4'-Methylenebis (2-Chloroaniline)
4,4'-Methylenedianiline
4,4'-Methylenedi-*o*-toluidine
4-Methyl-*m*-phenylenediamine
2-Naphthylamine
5-Nitro-*o*-toluidine
4,4'-Oxydianiline
4,4'-Thiodianiline
o-Toluidine
2,4,5-Trimethylaniline

Source: Reife *et al.*, 1999.

2.2 Dye classification

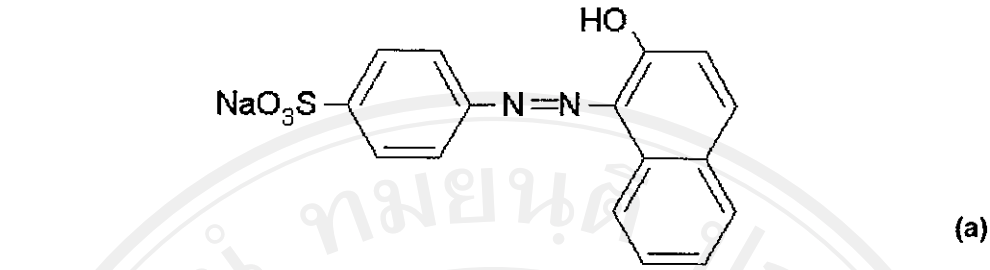
Colorants are divided to dyes and pigments. Dyes are soluble at some stage of the application process, whereas pigments, in general, retain essentially their particulate or crystalline form during application. A dye is used to impart color to materials of which it becomes an integral part. An aromatic ring structure coupled with a side chain is usually required for resonance and thus to impart color. Resonance structures that cause displacement or appearance of absorption bands in the visible spectrum of light are responsible for color.

Correlation of chemical structure with color has been accomplished in the synthesis of dye using a chromogen-chromophore with auxochrome. Chromogen is the aromatic structure containing benzene, naphthalene, or anthracene rings. A chromophore group is a color giver and is represented by the following radicals, which form a basis for the chemical classification of dyes when coupled with chromogen. Azo (–N=N–), carbonyl (=C=O), carbon (=C=C=), carbon-nitrogen (=C=NH or –CH=N–), nitroso (–NO) and nitro ($–NO_2$) are some examples of chromophore. The chromogen-chromophore structure is often not sufficient to impart solubility and cause adherence of dye to fiber. The auxochrome or bonding affinity groups such as amine, hydroxyl, carboxyl, and sulfonic radicals or their derivatives is necessary.

Based on chemical structure, dyes can be classified (review in five important classes) as the following according to Zollinger (1991) and Gregory (1990).

2.2.1 Azo dye class

Azo dyes contain at least one azo group (–N=N–), but possible to contain two, three, or, more rarely, four azo groups. The azo groups are mainly bound to benzene or naphthalene rings, but in some cases they are also attached to aromatic heterocycles or enolizable aliphatic groups. If any dye structures contained azo groups, as Figure 2.2, they can be grouped in azo dyes class immediately.



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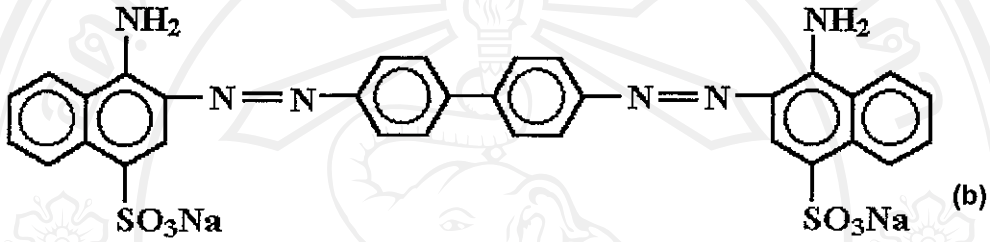

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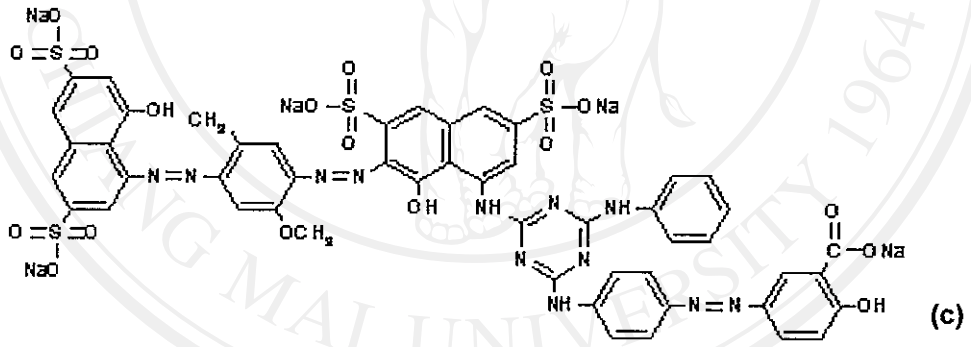

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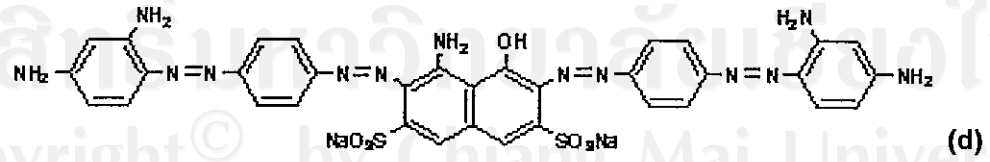


(d)

Figure 2.2 Structure of azo dyes

(a) Orange II (monoazo)

(b) Congo red (disazo)

(c) Direct green 26 (trisazo)

(d) Direct black 19 (tetrakisazo)

2.2.2 Anthraquinone dye class

Basic structure of anthraquinone dyes are shown in Figure 2.3a. Anthraquinone dyes are based on 9 and 10 positions of anthraquinone ring, which is essential, but colorless. To obtain any color, powerful electron donor groups such as amino or hydroxy are introduced into the structure. Color can be developed by intramolecular hydrogen bonding as showed in Figure 2.3b. Figure 2.3c is an example of this dye class.

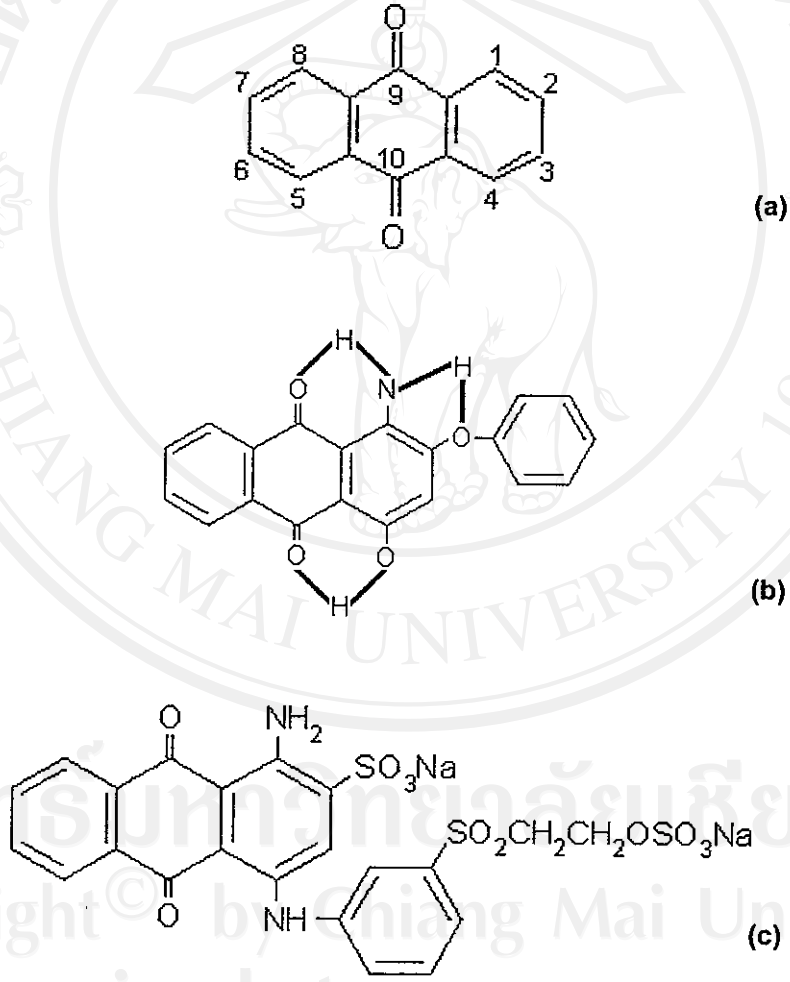


Figure 2.3 Structure of anthraquinone dyes

(a) Basic structure

(b) Intramolecular hydrogen bonding (thick lines)

(c) Remazol Brilliant Blue R

2.2.3 Indigoid dye class

Like the anthraquinone, indigoid dyes also contain carbonyl groups (=C=O). An identity of the dyes is H-chromogen performing blue color (as Figure 2.4a, H-chromogen look like a capital H). For example, indigo (Figure 2.4b) is used exclusively for dying denim jeans and jackets. In Thailand this dye is called "Kram".

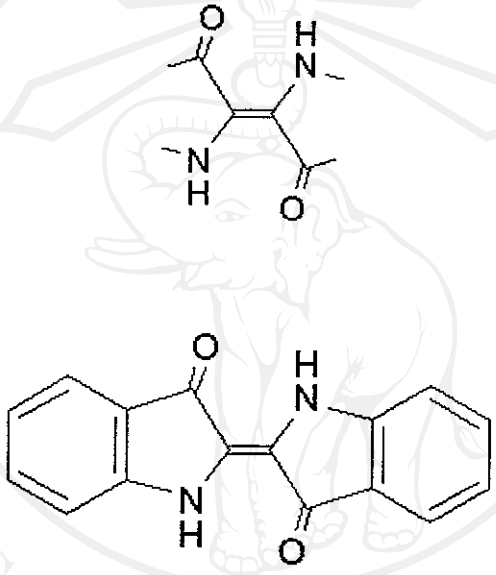


Figure 2.4 Structure of indigoid dyes

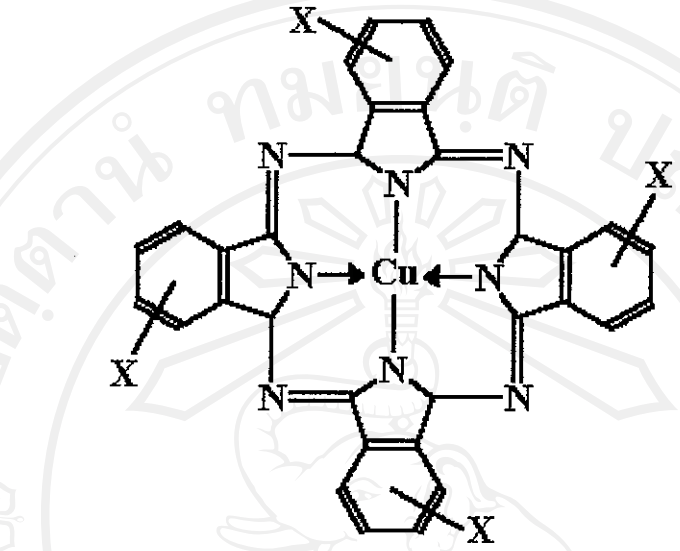
(a) H-chromogen of indigoid dyes

(b) Indigo

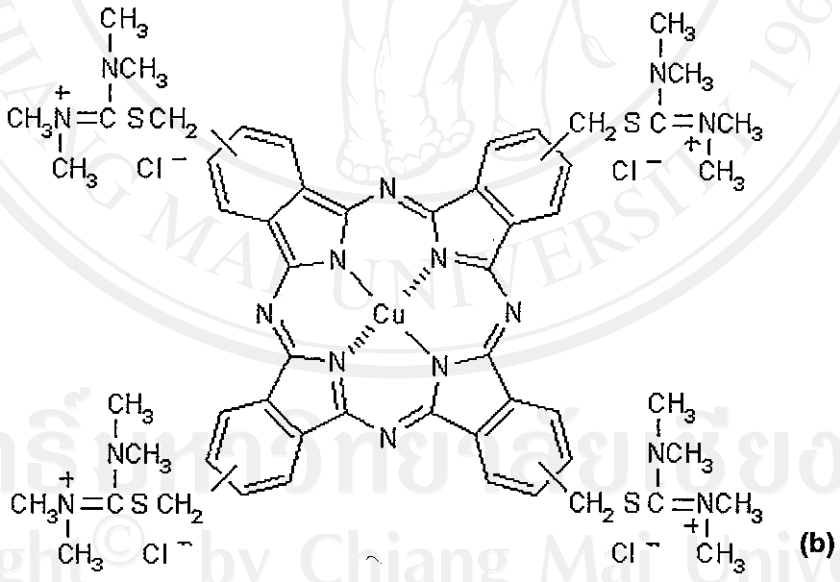
2.2.4 Phthalocyanine dye class

Phthalocyanine dyes have a complex structure with copper as a central atom. They have a general structural formula as Figure 2.5a, at the point marked X various groups are attached according to each specific dye. Phthalocyanine are analogs of the natural pigments such as chlorophyll which have magnesium as a central atom, while copper give a best combination of color and properties and consequently the majority of

phthalocyanine dyes are based on copper phthalocyanine. However, they hues are restricted to only blue and green such as Alcian blue (Figure 2.5b).



(a)



(b)

Figure 2.5 Structure of phthalocyanine dyes

(a) General structure

(b) Alcian blue

2.2.5 Arylmethane dye class

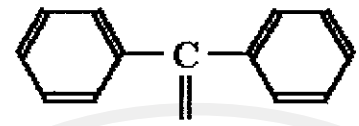
Arylmethane (Phenylmethane) dyes are derived from methane and divided to diarylmethane and triarylmethane dyes (basic structures are shown in Figure 2.6a and b, respectively). Diarylmethane dyes have two aryl rings such as Auramine O (Figure 2.6c). The chromophore is the carbon to nitrogen double bond. Triarylmethane dyes contain three aryl rings. These dyes also contain amino groups, and for that reason are sometimes identified as aminotriarylmethane dyes. In Acid fuchsin (Figure 2.6d), each of the three aryl rings has an amino group and quinoid ring is the chromophore.

2.3 Dye contaminated wastewater treatments

In entire groups of dyes, azo dyes are largest dye group (over 60-70% of all commercial dyes; Fu and Viraraghavan, 2001; van der Zee *et al.*, 2001) followed by anthraquinone and indigoid dyes (Wang and Yu, 1998). In the case of azo dyes, they occur in textile dyehouse wastewater about 5-1,500 ppm (Gottlieb *et al.*, 2003). When there are any dyes contaminating into wastewater, several choices of dye removal can be selected to solve the problems (reviewed by Knapp *et al.*, 2001; Robinson *et al.*, 2001a; Reife *et al.*, 1999; Banat *et al.*, 1996). The following paragraphs are classification of dye removal methods (highlight in biological treatments).

2.3.1 Physical treatments

1. Adsorption, adsorbents used such as activated carbon, silica gel, wood chips, fly ash, coal, microbial biomass and etc.
2. Membrane filtration including ultrafiltration and reverse osmosis.
3. Ion exchange, use of ion-exchanger resins.
4. Ionizing radiation or irradiation, use of short wave length radiating to dissolved oxygen forming powerful oxidizing free radicals attacking dye molecules.
5. Incineration.



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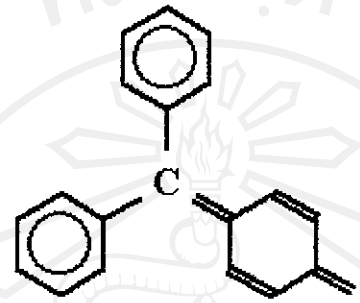

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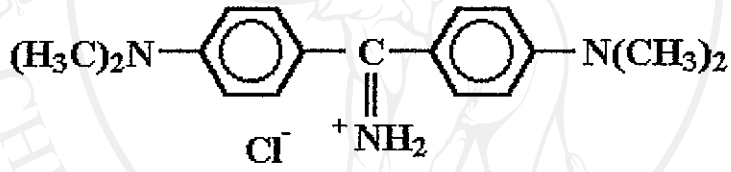

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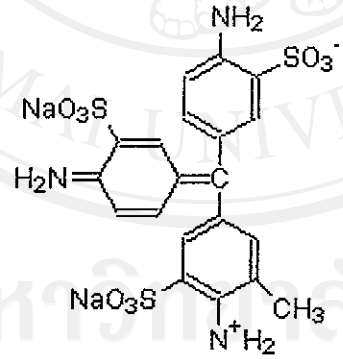

(d)

Figure 2.6 Structure of arymethane dyes

(a) Basic structure of diarymethane

(b) Basic structure of triarymethane

(c) Auramine O, diarymethane

(d) Acid fuchsin, triarymethane

2.3.2 Chemical treatments

1. Chemical oxidation, this method is use of oxidizing agents attacking dye molecules. The oxidizing agents used such as hydrogen peroxide, ozone, sodium hypochloride and etc.
2. Chemical flocculation, for example, ferrous-calcium hydroxide combination can be used.
3. Sometime, oxidizing agents and flocculates may be used together, for example, Fenton's reagent (ferrous-hydrogen peroxide).

2.3.3 Physicochemical treatments

For example, photochemical (UV-hydrogen peroxide), this method is used of hydrogen peroxide activated by ultraviolet light resulting the powerful hydroxy radicals, which capable of destroying the dye molecules.

2.3.4 Biological treatments

2.3.4.1 Conventional treatments

Conventional wastewater treatment plants for domestic and industrial wastewater such as stabilization ponds, aerated lagoons, trickling filters, activated sludge, anaerobic digestions and rotating biological contractors, can be used in dye removal occasionally. Those plants contain several microorganisms under the open systems that suitable to remove organic substances in wastewater more than complex recalcitrant structure molecules such as dyes (Fu and Viraraghavan, 2001; Knapp *et al.*, 2001) and aromatic amines, which can be formed via anaerobic treatment (Yesilada *et al.*, 2003).

2.3.4.2 Candidate microorganisms

Algae, bacteria and fungi have been investigated in dye decolorization in previous many reports and summarized in Banat *et al.* (1996), however, microorganisms which are reported that they gave a high efficiency in dyes decolorization are fungi, especially white rot fungi. Mechanisms of decolorization in this way can be possible in two means, dyes are degraded by the action of ligninolytic enzymes and adsorbed by the biomass. When a candidate microorganism was screened, it can be used in decolorization via some suitable plants or bioreactors and *in situ* remediation.

2.3.4.3 Enzymatic treatments

The potential advantages of enzymatic treatment compared to fungal or bacterial treatments are mainly associated to several factors including shorter treatment periods, operation at high and low contaminant concentrations, absence of delays associated with the lag phase of biomass, reduction in sludge volume (no biomass generation) and the simplicity of the process control (Lopez *et al.*, 2002).

Ligninolytic enzymes are extensively studied by several researchers for using in the decolorization. Those include lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. All three enzymes are oxidative enzymes that can oxidize dye molecules via co-substrates (mediators or free radicals). Useful of enzymes in biological treatments would be occurred when continuous immobilized enzyme reactors were completely constructed, but they are complex and costly.

2.3.5 Disadvantages

Although there are many methods for dyes decolorization, but each methods are different in disadvantages as the following:

1. Chemical, physical and physicochemical treatments are very expensive methods, they consume large amounts of chemicals, materials and energy.

2. Sludge generation in some methods such as membrane filtration and chemical flocculation cause a disposal problem (toxicity of sludge).
3. Chemical treatments, sometime, they raise much more problem from adding of several chemicals into wastewater.
4. Many conventional biological treatments such as activated sludge systems can not give a satisfied color removal.
5. Some methods such as using of sodium hypochloride and anaerobic digestion can produce toxic by-products.
6. Condition and contamination control are necessary when specific monomicrobial cultures were used.
7. When ligninolytic enzymes were used, they need enzyme preparation, enzyme immobilization, enzyme reactor construction and, especially, addition of small molecule co-substrates increasing treatment cost.

2.3.6 Alternative methods

As several reasons above, white rot fungi are an alternative and attractive way for dyes decolorization, but this still need more research and development to apply in dyes contaminated wastewater treatments. White rot fungi, which were reported to give a high efficiency in dye decolorization are *Phanerocheate chrysosporium*, *Pleurotus ostreatus*, *Coriolus versicolor* and others. Dyes can be degraded by their strong activity of non-specific ligninolytic enzymes.

2.4 White rot fungi

White rot fungi are a physiological rather than taxonomic grouping. They are the one in wood rotting fungi, the others are soft rot fungi and brown rot fungi according to their mode of attack and conditions in which they grow (Deacon, 1997). Soft rot and brown rot fungi degrade celluloses and hemicelluloses but have a little or no effect on lignin in wet and dry wood, respectively, while white rot fungi degrade celluloses, hemicelluloses and lignin more or less simultaneously.

2.4.1 Characteristics and Ecology

White rot fungi are filamentous higher fungi that inhabit the wood of dead and dying trees, most are members of the Basidiomycota but some are in the Ascomycota. The characteristic feature of white rots is their ability to degrade lignin within lignocellulosic substrates. White rots are so-called because of the white or bleached appearance of the rotted wood meaning lignin were completely degraded (Figure 2.7).

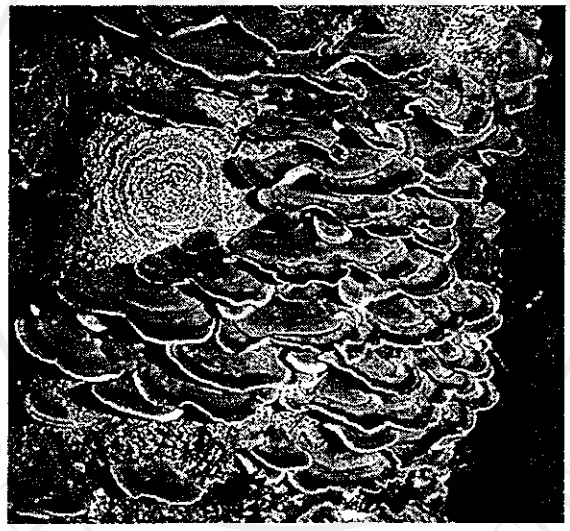

Figure 2.7 White area of wood degraded by white rot fungi (*Coriolus versicolor*)

2.4.2 Application in bioremediation

White rot fungi have the ability to degrade many xenobiotic compounds with a wide variety of structures. The potential for using of white rot fungi to treat pollutants and bioremediate contaminated land has been reviewed (Gradd, 2001; Pointing, 2001). Perhaps the most important thing to observe is the wide range of structures involved, most are aromatic structures (Table 2.2).

Table 2.2 Environmental pollutants degraded by white rot fungi

Type	Examples
Chlorinated aromatic compounds	Chlorophenols [e.g. pentachlorophenols (PCP), trichlorophenols (TCP), and dichlorophenols (DCP)]; Chlorolignols; Chloroguaiacols; 2,4-Dichlorophenoxyacetic acid (2,4-D); 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T); Polychlorinated biphenyls (PCBs); Dioxins; Chlorobenzenes
Dyes	Azure B; Congo red; Disperse Yellow 3; Orange II; Poly R; Reactive black 5; Reactive orange 96; Reactive violet 5; Remazol Brilliant Blue R; Solvent yellow 14; Tropaeolin
Nitroaromtics	2,4,6-Trinitrotoluene (TNT); 2,4-Dinitrotoluene; 2-Amino-4,6-dinitrotoluene; 1-Chloro-2,4-dinitrotoluene; 2,4-Dichloro-1-nitrotoluene; 1,3-Dinitrobenzene
Pesticides	Alachlor; Aldrin; Chlordane; 1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane (DDT); Heptachlor; Lindane; Mirex; Atrazine
Phenols	Phenol; *p*-Cresol
Polycyclic aromatic hydrocarbons	Anthracene; 2-Methyl anthracene; 9-Methyl anthracene; Benzo [*a*]pyrene; Fluorene; Naphthalene; Acenaphthene; Acenaphthylene; Phenanthrene; Pyrene; Biphenylene
Others	Benzene; Toluene; Ethylbenzene; *o*-, *m*-, *p*-Xylenes (BTEX compounds); Linear alkylbenzene sulfonate (LAS); Trichloroethylene

Sources: modified from Knapp *et al.*, 2001; and Reddy and Mathew, 2001.

Most early research on biodegradation by white rot fungi employed *P. chrysosporium*. However, In recent years, a much wider range of organisms has been studied both in terms of biodegradation of xenobiotics in general and colored materials in particular (Table 2.3). Additionally, many unidentified isolates have been used since it is

difficult to identify basidiomycetes from cultured mycelium, which often does not produce asexual spores or have any obvious distinguishing features. *P. chrysosporium* is undoubtedly the most commonly studied organism, with *C. vesicolor* clearly second favourite. *P. ostreatus*, *Bjerkandera adusta* and *Lentinus edodes* are also frequently used, with other species being studied to a much lesser extent.

2.4.3 *Coriolus versicolor*

Agaricus versicolor, *Bolentus versicolor*, *Coriolus versicolor*, *Polyporus versicolor*, *Polystictus versicolor, Poria versicolor* and *Trametes versicolor* are synonyms for the same organisms (Cui and Chisti, 2003; Knapp *et al.*, 2001; Cassland and Jonsson, 1999; Archibald *et al.*,1997) and their common names are Turkey tail fungi, Yun Zhi (Chinese), Dawaratake (Japanese) and Het Hing or Het Kra Dang or Het Rub Pat (Thai).

The visible form of *C. versicolor* are a fan-shaped mushroom with wavy margin and colored concentric zones. *C. versicolor* are obligate aerobes belonging to basidiomycete white rot fungi that are commonly found year-round on dead logs, stumps, tree trunks, and branches which are low nitrogen content (C:N ratio in wood is about 200:1-1,000:1; Evans and Hedger, 2001).

Low nitrogen content condition induces the ligninolytic enzyme production of the fungus, that show an important role to degrade lignin in the wood to obtain nitrogen for surviving. *C. versicolor* seem to use conventional cellulase enzyme for wood decay, but they are extremely efficient in their use of nitrogen. Deacon (1997) reported that the hyphea of *C. versicolor* have been found to have a nitrogen content of 4% when grown on laboratory media of C:N ratio, 32:1; but they has only 0.2% nitrogen content when grown on a medium of C:N, 1,600:1. In nitrogen-poor conditions this fungus may preferentially allocate nitrogen to the production of extracellular enzymes and essential cell components, and also efficiently recycle the nitrogen in their mycelia.

C. versicolor can produce all three major ligninolytic enzymes; LiP, MnP, and laccase (Evan and Hedger, 2001; Archibald *et al.*, 1997), but laccase are produced strongly than other 2 peroxidases (Cerniglia and Sutherland, 2001; Cohen and Hadar, 2001; Fu and Viraraghavan, 2001).

Table 2.3 White rot fungi used in biodegradation or decolorization studies

Bjerkandera adusta and *Bjerkandera* spp.
Ceriporia metamorphosa
Chrysonilia sitophila
Chrysosporium lignorum
Cyathus bulleri and *Cyathus* spp.
Daedalea flavida
Daedaleopsis confragosa
Dichomitus squalens
Flammulina velutipes
Funalia trogii
Ganoderma lucidum
Geotrichum candidum
Hericium erinaceum
Irpex lacteus
Lentinus (Lentinula) edodes and *L. tigrinus*
Merulius tremellosus
Mycoacia nothofagi
Phanerocheate chrysosporium, P. flavido-alba and *P. sodida*
Phellinus gilvus and *P. pseudopunctatus*
Phlebia brevispora, P. fascicularia, P. floridensis, P. radiata and *P. tremellosa*
Piptoporus betulinus
Pleurotus eryngii, P. ostreatus and *P. sajor-caju*
Polyporus ciliatus, P. frondosus and *P. sanguineus*
Pycnoporus cinnabarinus and *P. sanguineus*
Schizophyllum commune
Stereum hirsutum and *S. rugosum*
Trametes (Coriolus) versicolor, T. hirsuta and *Trametes* spp.

Source: modified from Wesenberg *et al.*, 2003; and Knapp *et al.*, 2001.

2.5 Ligninolytic enzymes

The ligninolytic enzymes (lignolytic or lignin modified or lignin degrading enzymes) are extracellularly excreted by the white rot fungi initiating the oxidation of lignin in the extracellular environment of the fungal cell. Because lignin is degraded in a non-specific, radical-based oxidation, ligninolytic enzymes are capable of degrading a mixture of various pollutants. The number of compounds knew to be degraded by the enzymes continues to increase with the ongoing research. The ligninolytic enzymes perform a one-electron oxidation, thereby generating cation radicals of the substrates. The cation radicals may undergo spontaneous chemical reaction such as C–C cleavage or hydroxylation resulting in more hydrophilic products. This is still complex process of oxidation, reduction, methylation and hydroxylation. Despite much research on the oxidative mechanism of the enzymes, the mechanism of lignin degradation and the oxidation of lignin-related compounds are not entirely understood. The reaction of the extracellular ligninolytic enzymes is quite complicated involving numerous low molecular weight cofactors that may serve as redox mediators (Mester and Tien, 2000).

White rot fungi variously secrete one or more of three extracellular ligninolytic enzymes that are essential for lignin degradation, and which combine with other processes to effect lignin mineralization. Table 2.4 shows various ligninolytic enzyme secretions by white rot fungi reviewed by Jarosz-Wilkolazka *et al.* (2002). The three enzymes comprise: laccase, lignin peroxidase, and manganese peroxidase. Some authors also report novel manganese independent peoxidase in some white rot fungi (Pointing, 2001).

2.5.1 Laccase

Laccase (copper-containing phenoloxidase; EC 1.10.3.2) is a member of the small group of proteins known as the blue multicopper oxidases. These proteins (laccase, ascorbate oxidase and ceruloplasmin) all contain four or more copper atoms and have the property of reducing dioxygen completely to water. The laccases of ligninolitic fungi are secreted glycoproteins with the ability to catalyse the one-electron oxidation of wide range of dihydroxy and diamino aromatic compounds (Harvey and Thurston, 2001).

In the catalytic cycle, laccase uses molecular oxygen and ABTS (2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate; artificial mediator) as a co-substrate. ABTS assist laccase to form couple reaction oxidizing various organic molecules, although they are not substrate of laccase directly. Figure 2.8 shows a catalytic cycle of laccase.

There are a few worker reporting about native mediators for laccase catalysis such as 3-hydroxyanthranilate identified from white rot fungus *Pycnoporus cinnabarinus* (Pointing, 2001).

2.5.2 Manganese peroxidase

Manganese peroxidase (Manganese dependent peroxidase, EC 1.11.1.13; MnP) is an extracellular gycosylated heme protein secreted by a variety of white rot fungi that uses hydrogen peroxide to oxidize Mn(II) to a Mn(III)-chelate which in turn oxidized phenolic substrates as a freely diffusible, non-specific oxidant. The heme is the site of oxidation of the protein by hydrogen peroxide, which is essential in creating the catalytic intermediates, termed Compound I and Compound II, that are required for catalysis. In MnP, there is a unique binding site for Mn(II) that involves the carboxylate side chains of three amino acid residues (Harvey and Thurston, 2001).

Reaction of native enzyme (E) with hydrogen peroxide yields Compound I (Equation 2.1). Two steps of single electron reduction by Mn(II) restore the native enzyme via the intermediate Compound II (Equation 2.2 and 2.3). Importantly for catalysis, the supply of hydrogen peroxide relative to Mn(II) needs to be poised to ensure that the competing reacting of Compound II with hydrogen peroxide does not take place (Equation 2.4), since this has the effect of driving the enzyme into a catalytically inactive mode.

$$E + H_2O_2 \longrightarrow \text{Compound I} + H_2O \quad (2.1)$$

$$\text{Compound I} + Mn(II) \longrightarrow \text{Compound II} + Mn(III) \quad (2.2)$$

$$\text{Compound II} + Mn(II) \longrightarrow E + Mn(III) + H_2O \quad (2.3)$$

$$\text{Compound II} + H_2O_2 \longrightarrow \text{Compound III (inactive)} \quad (2.4)$$

Table 2.4 Various ligninolytic enzymes secreted by white rot fungi

Speicies	Source[a]	Presence of		
		Laccase	LiP	MnP
Abortiporus biennis	BIUR	+	ND	ND
Bjerkandera fumosa	BIUR	+	ND	ND
Cerrena unicolor	BIUR	+	-	+
Clitocybula dusenii	IMUJ	+	+	+
Flammulina velutipes	FCTUA	+	-	ND
Ganoderma applanatum	FCTUA	+	ND	ND
Ganoderma lucidum	FCTUA	+	-	+
Keuhneromyces mutabilis	CBS	+	+	+
Lentinus edodes	FCTUA	+	-	+
Nematoloma frowardii	IMUJ	+	+	+
Panus tigrinus	BIUR	+	+/-	+
Perenniporia subacida	FCTUA	+	ND	ND
Phanerochaete chrysosporium	BIUR	+	+	+
Phlebia radiata	ATCC	+	+	+
Pholiota glutinosa	FCTUA	+	ND	ND
Pleurotus pullmonarius	BIUR	+	+	+/-
Pycnoporus coccineus	FCTUA	+	ND	ND
Stropharia rugosoannulata	ITAT	+	-	+
Trametes sanguinea	FCTUA	+	ND	+
Trametes (Coriolus) versicolor	FPD	+	+	+

Note: [a] ATCC – American Type Culture Collection; BIUR – Botanisches Institut II der Universitat Regensburg, Germany; CBS – Centralbureau voor Schimmelcultures, Baarn, Holland; FCTUA – Forest Products Chemistry Laboratory, Tokyo University of Agriculture, Japan; FPD – Forest Pathology Department, Agriculture Academy, Cracow, Poland; IMUJ – Institut fur Mikrobiologie, Friedrich-Schiller Universitat, Jena, Germany; ITAT – Institute of Technology and Chemical Engineering, Technical Agricultural Academy, Bydgoszcz, Poland.

Source: modified from Jorosz-Wilkolazka *et al.*, 2002.

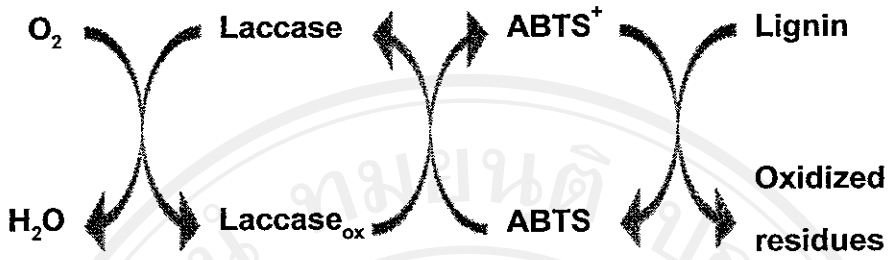

Figure 2.8 Catalytic cycle of laccase

Source: modified from Bajpai *et al.*, 1999.

Organic acids such as malonate, citrate, glyoxylate and oxalate are essential in chelating and stabilizing Mn(III) and are common secondary metabolites of wood rotting basidiomycetes, secreted at the same time as MnP. Among these, oxalate shows unique effects in chelating and stabilizing Mn(III) and may bind quite closely to the heme during catalysis (Harvey and Thurston, 2001). The catalytic cycle of MnP is shown in Figure 2.9.

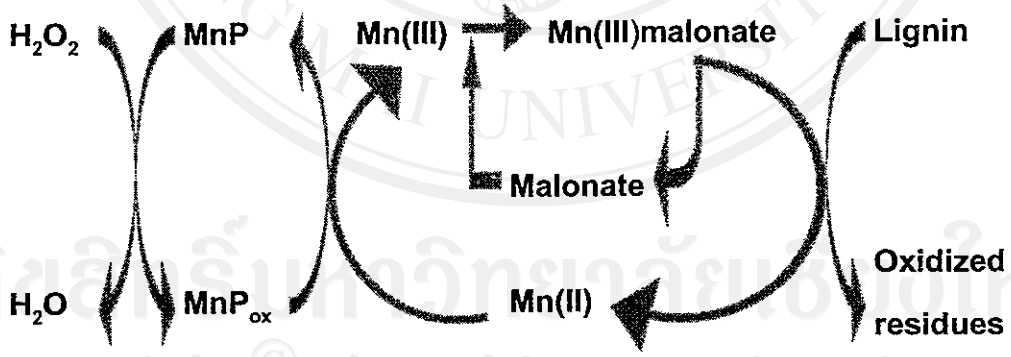

Figure 2.9 Catalytic cycle of manganese peroxidases

Source: modified from Archibald *et al.*, 1997.

2.5.3 Lignin peroxidase

Lignin peroxidase (ligninase; EC 1.11.1.14; LiP) is also an extracellular glycosylated heme protein depending on hydrogen peroxide for catalysis. LiP is unique in being able to produce radical cations from the one-electron of non-phenolic aromatic compounds such as veratryl alcohol (VA) which have redox potentials beyond the reach of either MnP or laccase. Radical cations of VA are able to act as non-specific redox mediators, with the effect that both the substrate range and redox capacity of LiP can be extended.

LiP has the same heme and similar active site residues as MnP and the same catalytic cycle. Redox potential, in part, determines whether an aromatic nucleus is a substrate for LiP. However, in much the same way that Mn(II) is a crucial substrate for MnP, small dimethoxylated non-phenolic aromatics such as VA serve as crucial substrates for LiP. The products of the oxidation of dimethoxylated aromatics are radical cations (Figure 2.10). VA is a fungal metabolite produced at the same time as LiP (Harvey and Thurston, 2001).

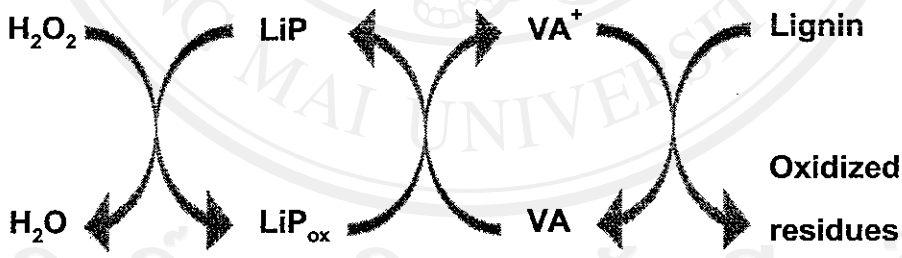

Figure 2.10 Catalytic cycle of lignin peroxidases

Source: modified from Harvey and Thurston, 2001.

2.5.4 Redox mediators

Direct and specific interactions between lignin (or recalcitract structural analogs) and ligninolytic enzymes are highly improbable without redox mediactors. Rather low

molecular weight, diffusible redox mediators provide high redox potentials to attack lignin and are able to migrate into the lignocellulose complex. Examples of native as well as synthetic mediators are given in Table 2.5. They could be involved in the ligninolytic enzyme catalysed generation of reactive radical moieties from a variety of lignin-like substrates, but also in the formation of reactive oxygen species which either directly or indirectly could attack lignin or xenobiotic molecules.

Organic acids, excreted by several fungi, chelate and stabilize Mn(III). MnP was found to simultaneously decompose organic acids (such as malonate) oxidatively and oxidize Mn(II) to Mn(III) even in the absence of H_2O_2. Thus, organic acids are postulated to be the origin of carbon-centered radicals (acetic acid radicals; $COOH–C^*H_2$; Equation 2.5), peroxyl radicals ($COOH–CH_2OO^*$; Equation 2.6), superoxide (O_2^{*-}; Equation 2.9 and 2.12), formate radicals (CO_2^{*-}; Equation 2.10 and 2.11). Such radicals could be source of peroxides, which can be used by MnP as substrates instead of H_2O_2. Consequently, even fungi obviously lacking H_2O_2-generating oxidases could be efficient lignin degraders and, by extension, useful in the degradation of xenobiotics such as dyes (Wesenberg *et al.*, 2003).

$$COOH–CH_2–COOH + Mn(III) \longrightarrow COOH–C^*H_2 + CO_2 + H^+ + Mn(II) \quad (2.5)$$

$$COOH–CH_2 + O_2 \longrightarrow COOH–CH_2OO^* \quad (2.6)$$

$$COOH–CH_2OO + Mn(II) \longrightarrow COOH–CH_2OOH + Mn(III) \quad (2.7)$$

$$COOH–CH_2OOH + 2Mn(II) \longrightarrow COOH–CHO + H_2O + 2Mn(III) \quad (2.8)$$

$$COOH–CH_2OO^* + O_2 \longrightarrow COOH–COOH + O_2^{*-} + H^+ \quad (2.9)$$

$$COOH–CHO + Mn(III) + \tfrac{1}{2}O_2 \longrightarrow HCOOH + CO_2^{*-} + Mn(II) \quad (2.10)$$

$$COOH–COOH + Mn(III) \longrightarrow CO_2 + CO_2^{*-} + Mn(II) \quad (2.11)$$

$$CO_2^{*-} + O_2 \longrightarrow CO_2 + O_2^{*-} \quad (2.12)$$

$$O_2^{*-} + Mn(II) + 2H^+ \longrightarrow H_2O_2 + Mn(III) \quad (2.13)$$

$$H_2O_2 + 2Mn(II) \longrightarrow H_2O + 2Mn(III) \quad (2.14)$$

Veratryl alcohol, a secondary metabolite of several white rot fungi, after its oxidation to VA-cation radical (VA^{+}) by LiP, acts as a mediator for the degradation of lignin. However, due to the short life span of VA^{+} long distance charge transfers are not likely to occur. Mediating properties of VA could be enhanced if the radical is somehow complexed to the LiP. Nevertheless, LiP is stimulated by VA probably by protecting the enzyme against the damaging effect of H_2O_2.

3-Hydroxyanthranilic acid (HAA; 2-Amino-3-hydroxybenzoic acid; Figure 2.11) was the first natural mediator for laccases described. This mediator enables a laccase catalyzed oxidation of nonphenolic lignin model dimers. To delignify kraft pulp by laccase a number of synthetic mediators have been tested. For instance, using ABTS laccases are able to attack nonphenolic lignin model compounds and to delignify kraft pulp. The discovery of 1-hydroxybenzotriazole (HBT), an effective laccase mediator in pulp processing lead to a new class of mediators with NOH as the functional group, which is oxidized to a reactive radical (R–NO). These mediators including 4-hydroxy-3-nitroso-1-naphthalenesulfonic acid (HNNS), 1-nitroso-2-naphthol-3,6-disulfonic acid (NNDS), and Remazol brilliant blue (RBB) have been shown to support delignification reactions by laccases (Wesenberg *et al.*, 2003).

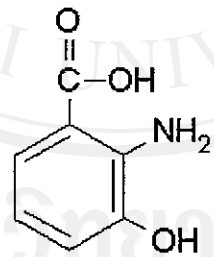

Figure 2.10 3-Hydroxyanthranilic acid

2.5.5 Manganese independent peroxidase

Manganese independent peroxidase (EC 1.11.1.7; MIP) was discovered in the conditions of no Mn(II) and VA, this novel heme peoxidase can present ligninolytic activity.

Moreira *et al.* (1998) demonstrated that the biobleaching activity of the white rot fungus *Bjerkandera* sp. strain BOS55 was not dependent on the presence of manganese. Even when kraft pulp was extracted free of manganese by EDTA, it was bleached as extensively as pulp supplemented with manganese. In spite of the lack of manganese, MnP of the BOS55 was found to be the major oxidative enzyme present while LiP and MIP were also present. The production of MnP by *Bjerkandera* sp. strain BOS55 in the absence of manganese nutrients is remarkable because most white rot fungi require manganese for *mnp* gene expression and protein production. The results suggested that under manganese-deficient conditions, MnP from *Bjerkandera* may have roles in pulp biobleaching together with MIP and LiP. Another report about MIP is available in Vyas and Molitoris (1995), they found MIP activity in white rot fungus *Pleurotus ostreatus*. However, there is no clearly catalytic mechanism of MIP have been reported.

Table 2.5 Some native and synthetic mediators in ligninolytic enzyme systems

Mediators	**Enzymes**	**Organisms**
Native mediators		
Mn(III)	MnP	*P. chrysosporium*
Organic acids (malonate, oxalate, etc.)	LiP, MnP	*P. chrysosporium, P. ostreatus, P. radiata,* etc.
Veratryl alcohol	LiP	*P. chrysosporium*
3-Hydroxyanthranilic acid	Laccase	*P. cinnabarinus*
2-Chloro-1,4-dimethoxybenzene	LiP	*C. versicolor*
Synthetic mediators		
1-Hydroxybenzotriazole (HBT)	Laccase	*C. versicolor, P. cinnabarinus, P. ostreatus,* etc.
Violuric acid	Laccase	*C. versicolor, P. cinnabarinus,* etc.
ABTS	Laccase	*C. versicolor, P. ostreatus,* etc.

Source: modified from Wesenberg *et al.*, 2003.

2.5.6 Associated enzymes

There are enzymes associating with ligninolytic system in lignin breakdown, but are unable to degrade lignin alone. Glyoxal oxidase (EC 1.2.3.5) and superoxide dismutase (EC 1.15.1.1) produce the hydrogen peroxide required by MnP and LiP. Other enzymes are involved in feedback mechanisms and serve to link lignocellulose degradation pathways. These comprise glucose oxidase (EC 1.1.3.4), aryl alcohol oxidase (EC 1.1.3.7), cellobiose: quinone oxidoreductase (EC 1.1.5.1), and cellobiose dehydrogenase (EC 1.1.99.18) (Pointing, 2001).

2.5.7 Application

In the same way of white rot fungi, application of their ligninolytic enzymes in waste treatment are similarity and have been reviewed in Mayer and Staples (2002), and Duran and Esposito (2000). There is growing recognition that enzymes can be used in many remediation processes to target specific pollutants for treatment. The potential advantages of the enzymatic treatment as compared with conventional treatments include: application to recalcitrant materials, operation at high and low contaminant concentrations over a wide pH, temperature and salinity range, no production of biomass and the easy control process among others. Ligninolytic enzymes used in waste treatment are summarized in Table 2.6.

2.6 Decolorization by white rot fungi

2.6.1 Decolorization of dark effluents

Not only textile dyes have been studied in decolorization by white rot fungi, but also other sources of dark color in water have been studied such as chemical industry effluents, cotton bleaching effluents, molasses wastewater, olive oil milling wastewater, and paper making and pulping effluents that conventional biological treatments have no effect on these colors (Knapp *et al.*, 2001). Some of them have no clear report in

decolorization mechanism while some are tend to be clear. However, ligninolytic enzymes are the keys stimulating degradation of the color giver structures.

In chemical industry effluents such as manufacture of nitrated stilbene sufonic acid contain an azo-linked chromophore while cotton bleaching effluents and molasses wastewater contain melanoidin pigments and other uncertain chromophores. Olive oil milling effluents and paper making and pulping effluents contain phenolic compounds and their derivatives which are recalcitrant and toxic. There are many reports in decolorization of those effluents by white rot fungi and their ligninolytic enzymes summarized in Table 2.7.

Table 2.6 Potential application of ligninolytic enzymes

Enzymes	Sources	Applications
Laccase	*Cerrena unicolor*	Phenol detoxification, 2,4-dichlorophenol degradation
	Pycnoporus cinnabarinus	Benzopyrenes degradation
	Pyricularia oryzae	Azo dyes degradation
	Trametes hispida	Dyes decolorization
	Trametes (*Coriolus*) *versicolor*	Textile effluent degradation, chlorophenols degradation, urea derivatives degradation
LiP	*Chrysonilia silophila*	Kraft effluent remediation
	Phanerochaete chrysosporium	Aromatic compounds degradation, phenolic materials degradation
MnP	*Lentinula edodes*	Chlorophenol degradation, herbicide (Diulon) degradation
	Nematoloma frowardii	Lignins degradation
	P. chrysosporium	Phenols and lignins degradation, pentachlorophenol degradation, dyes degradation

Source : modified from Duran and Esposito, 2000.

Table 2.7 Decolorization in various effluents by white rot fungi and their enzymes

Effluents	**White rot fungi**	**Methods used**
Chemical industy	*Coriolus versicolor*	Culture
	Phanerochaete chrysosporium	Culture
	Pleurotus ostreatus	Culture
Cotton bleaching	*C. versicolor*	Culture, crude enzyme
Molasses	*C. versicolor*	Fed batch culture, continuous decolorization, MnP
	Mycelia sterilia	Repeated batch culture
	P. chrysosporium	Culture
Olive milling	*C. versicolor*	Culture, immobilized culture
	Dichomitus aqqualens	Culture
	Funalia trogii	Culture
	Lentinus edodes	Repeated batch culture, Immobilized repeated batch culture
	P. chrysosporium	LiP and MnP
	Phanerochaete flavido-alba	Laccase, MnP
	Phlebia radiata	Culture
	P. ostreatus	Culture, crude enzyme
	Polyporus frodosus	Culture
Pulping	*C. versicolor*	Packed bed reactor, immobilized fluidized bed reactor
	Merulius tremellosus	Culture
	P. chrysosporium	Immobilized packed bed reactor, hollow fibre reactor
	Phlebia radiata	Culture

Source: modified from Lacina *et al.*, 2003; Fu and Viraraghavan, 2001; and Knapp *et al.*, 2001.

2.6.2 Dye decolorization

Recent publications on the decolorization of dyes by white rot fungi and/or their enzymes are shown in Table 2.8.

Table 2.8 Dye decolorization by white rot fungi and their enzymes

Dye classes	White rot fungi	Methods used	Mechanisms
Azo	*Bjerkandera adusta*	Culture, MnP	MnP
	Choriolus versicolor	Culture, LiP	Biodegradation, LiP, laccase, adsorption
	Phanerochaete chrysosporium	Culture, column bioreactor, fixed film bioreactor, peroxidases, LiP	Biodegradation, peroxidases, LiP
	Pleurotus eryngii	MnP	MnP
	Pleurotus ostreatus	Culture, peroxidases	Peroxidases
	Polyporus ostreiformis	Culture, LiP	LiP
	Pycnoporus cinnabarinus	Laccase	Laccase
	Pyricularia oryzae	Laccase	Laccase
	Trametes hispida	Culture	-
Anthraquinone	*B. adusta*	Culture	-
	C. versicolor	Culture, LiP	Biodegradation, LiP, laccase, adsorption
	P. chrysosporium	Culture, packed bed bioreactor, LiP	LiP
	P. ostreatus	Culture	-
	P. cinnabarinus	Packed bed bioreactor	Laccase
	T. hispida	Culture	-

Table 2.8 (Continued)

Dye classes	White rot fungi	Methods used	Mechanisms
Indigoid	*C. versicolor*	Culture, LiP	Biodegradation, LiP, laccase, adsorption
	P. chrysosporium	Culture, LiP	LiP
Phthalocyanine	*B. adusta*	Culture, MnP	MnP
	C. versicolor	Culture, LiP	LiP
	P. chrysosporium	Culture, LiP	LiP
	P. eryngii	MnP	MnP
	P. ostreatus	Culture	-
	T. hispida	Culture	-
Arylmethane	*C. versicolor*	Culture	-
	Cyathus bulleri	Culture, laccase	Laccase
	P. chrysosporium	Culture, column bioreactor, LiP	LiP
	P. ostreatus	Culture, peroxidases	Peroxidases
	P. cinnabarinus	Laccase	Laccase
Polymeric	*C. versicolor*	Culture	-
	Chrysosporium lignorum	Culture, immobilized culture	-
	C. bulleri	Culture	Laccase
	P. chrysosporium	Culture, LiP	LiP
	P. ostreatus	Peroxidases	Peroxidases
Heterocyclic	*C. versicolor*	Culture	-
	P. chrysosporium	Culture, LiP	LiP
	P. ostreatus	Culture, peroxidases	Peroxidases

Source: modified from Knapp *et al.*, 2001; and Fu and Viraraghavan, 2001.

2.6.3 Dye decolorization mechanisms

Dyes can be eliminated or disappeared from treated solution or wastewater of white rot fungi by two mechanisms, action of ligninolytic enzymes and adsorption to their biomass as the detail below.

2.6.3.1 Biodegradation

Biodegradation is the major mechanism in dye decolorization by white rot fungi (Table 2.8) because they can produce ligninolytic enzymes to mineralize the dyes. However, the relative contributions of laccase, MnP and LiP to the decolorization of dyes may be different for each fungus. For the fungus *P. Chrysosporium*, LiP played a major role in dye decolorization (Table 2.8).

Pathways for degradation of azo dyes by peroxidase from *P. chrysosporium* have been investigated. The machanism propose sequential abstraction of two electrons by peroxidase action followed by attack by water, which results in cleavage of the diazo linkage. Cleavage of the azo dye can occur asymmetrically to give a quinone and phenyl diazine (Figure 2.12). The latter decomposes as a result of attack by oxygen to give nitrogen and a phenyl compound or a sulfophenyl hydroperoxide depending on the type of dye involved (Knapp *et al.*, 2001).

A similar pathway has been reported in degradation of phenolic dyes by laccase. Azo bond can be split symmetrically during peroxidase attack, one nitrogen remaining attached to each aromatic ring. This would give rise to compounds such as amino or nitroso-substituted aromatics or quinone imines. When LiP is utilized, VA has been shown to stimulate decolorization. Many authors consider the peroxidases to be of prime importance in decolorization of dyes while laccase have also been shown to catalyse certain decolorizatins (Knapp *et al.*, 2001).

For *C. versicolor*, dye decolorization was dependent on dye structures. Anthraquinone dye was an laccase substrates directly while azo and indigoid dyes were not the substrates of laccase (Wong and Yu, 1999). Pathway of indigo dye degradation by laccase has been demonstrated by Campos *et al.* (2001) that laccases used in their experiment was produced and purified from *Trametes hirsuta* and *Sclerotium rolfsii*.

Oxidation of indigo to isatin is depicted in Figure 2.13. Since the actual catalytic activity of laccase involves the step-wise abstraction of four electrons from the substrate, it seems logical that the first steps in indigo oxidation proceed analogous to the electronchemical oxidation, which has been shown to result in the formation of dehydroindigo. This compound may be easily attacked by nucleophiles like water molecules, which leads to the incorporation of oxygen atoms into the decomposition products.

2.6.3.2 Biosorption

In addition to biodegradation, a biosorption mechanism might also play a role in decolorization of dyes by fungi. Knapp *et al.* (1995) reported that the extent of color removal by adsorption was always limited, generally less than 50%. In the case of *C. versicolor*, adsorption accounted for only 5-10% of color removal (Fu and Viraraghavan, 2001). However, the adsorption level is depending on type of microorganisms and their environmental condition.

In some fungi, biosorption is the only decolorization mechanism, but with white rot fungi both adsorption and degradation can occur. The relative importance of each mechanism can be difficult to assess since it is difficult to find suitable controls that contain enzymically inactive mycelium. Heat-killed mycelia have been used but their adsorptive properties can differ from those of live mycelia. Treatment with biocides (e.g. mercuric chloride or sodium azide) can kill the mycelium but do not guarantee that the enzyme complement will be inactivated. Furthermore, some biocides can complex with certain chromophores. In some studies, controls have not been included to account for adsorption. However, this may not always be necessary since if color removal is extensive then visual examination of the fungal biomass is often enough to confirm whether significant adsorption has occurred (Knapp *et al.*, 2001).

With white rot fungi, adsorption does not appear to be the principal mechanism of decolorization. It is likely that adsorption can play apart in the overall process, since prior adsorption to fungal mycelium may serve to bring chromophores onto closer contact with the degradative enzymes, which are often largely associated with the cell surface. After initial adsorption, oxidative degradation will then be occurred (Wang and Yu, 1998).

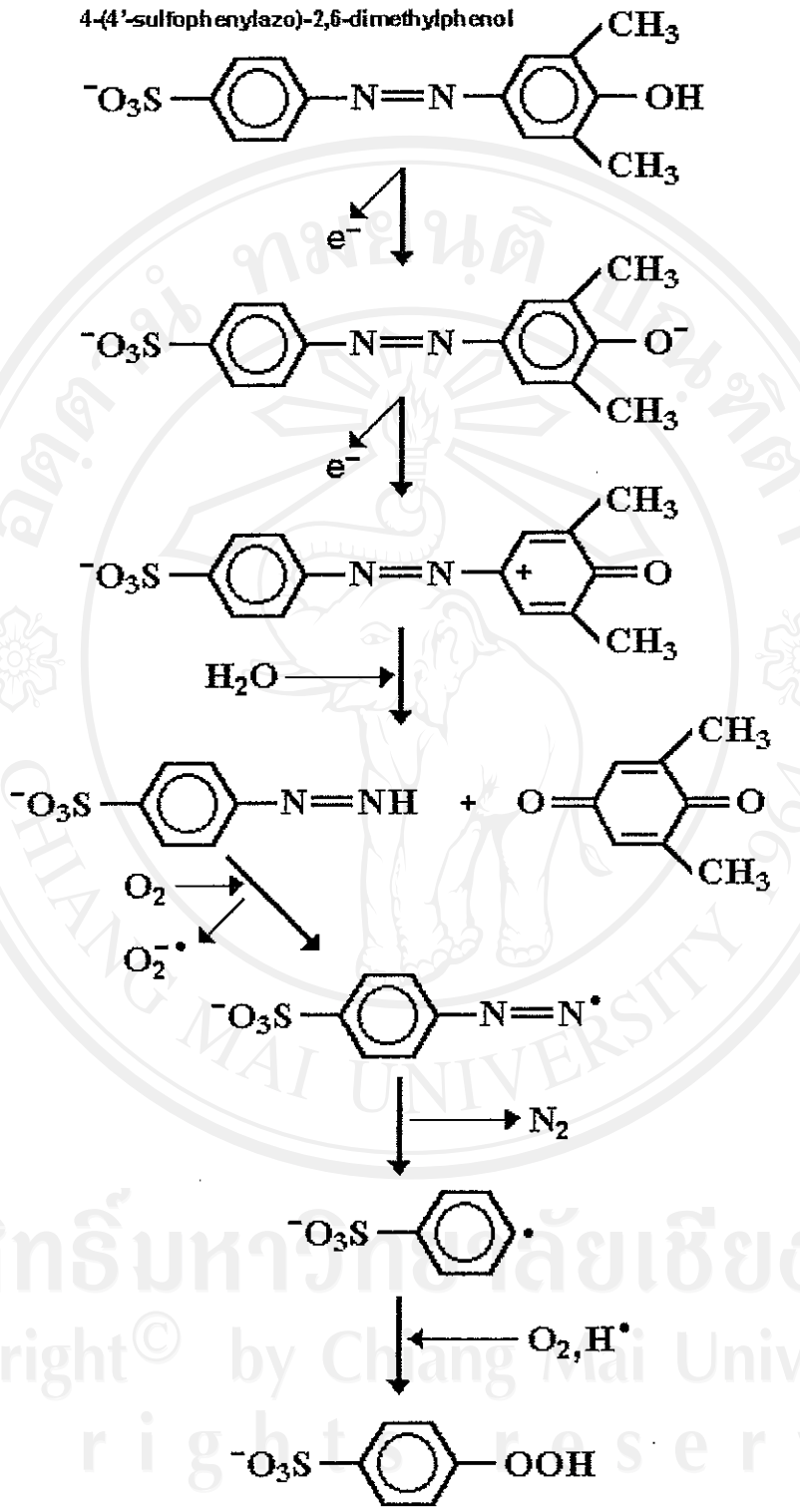

Figure 2.12 Possible pathway of azo dye degradation

Source: modified from Knapp *et al.*, 2001.

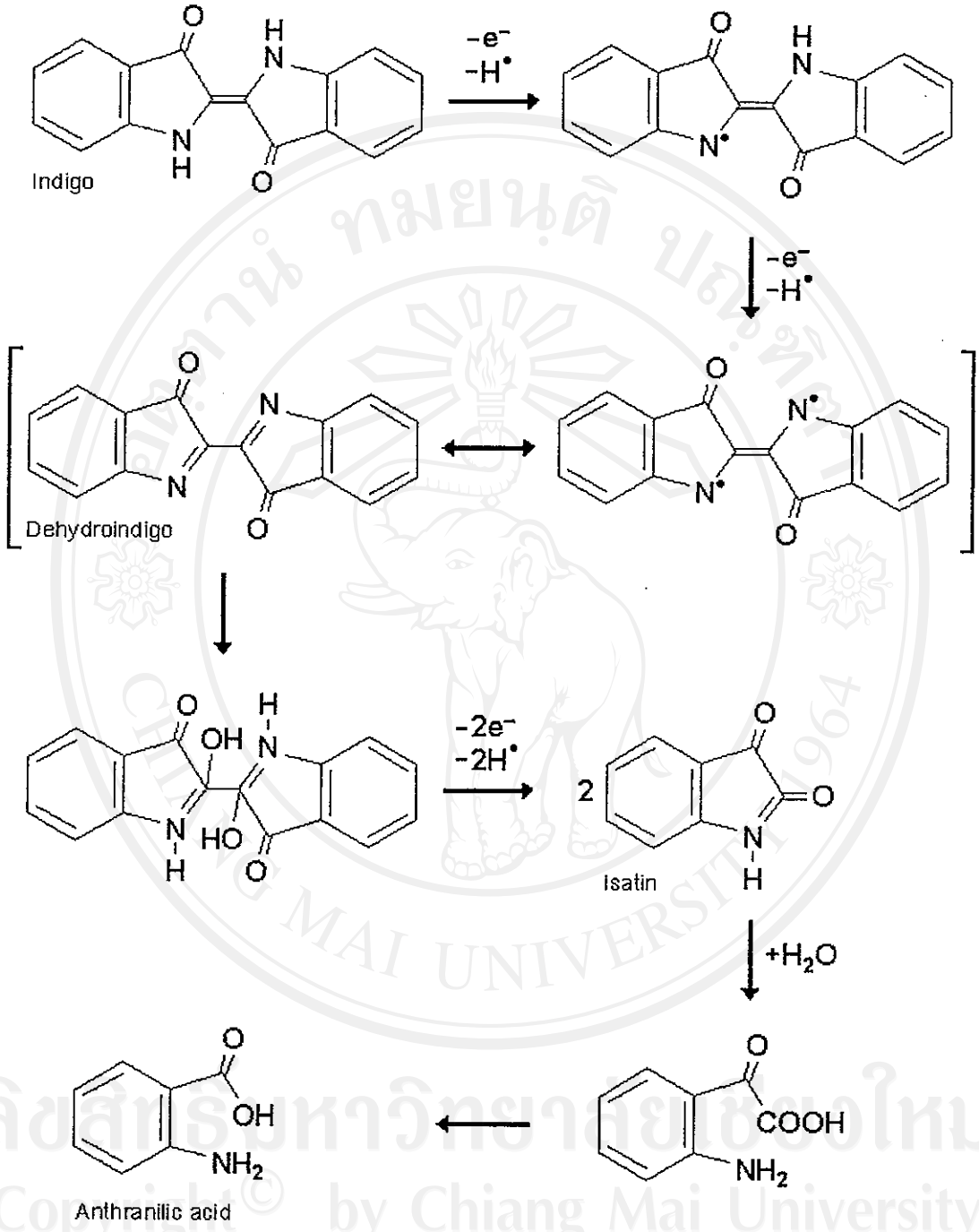

Figure 2.13 Possible pathway of indigo dye degradation

Source: Campos *et al.*, 2001.

2.7 Immobilized fungal bioreactor

When effective microorganisms in specific purpose such as enzyme production and pollutant remediation have been isolated and screened, they might be manipulated to raise their specific abilities and improved some weaknesses. After that, almost, where the microorganisms are used is the vessels called bioreactors or plants. Cell immobilization give several advantages for use in bioreactor such as operating with high cell density, easy to separate cell from product, and easy in continuous mode operation and cell recycling.

2.7.1 Cell immobilization

Numerous cell immobilization methods have been demonstrated. In principle, the methods can be divided into two major categories based on active and passive techniques. Active immobilization can be accomplished by various chemical agents, whereas passive immobilization occurs when films or flocs of cells form naturally around or within support material provided for that purpose or when cell adhesion on solid surface occurs through electrostatic interaction.

Popular method for fungal immobilization is colonization using suitable porous materials. However, colonization, passive immobilization, can be used in wide variety of cell types as Table 2.9. Porous materials used are essentially an interconnecting void within an open network of matrix support. Unlike technique involving active immobilization, the use of porous supports does not require the growth of cells prior to immobilization. The inert porous particles are simply placed in the vessel before sterilization, and the vessel is inoculated in the normal way. Cells become immobilized within the support as a natural consequence of growth during an initial batch period. This technique has been applied successfully to a wide variety of purpose (Table 2.9). Many kind of porous supports such as polyester, polyether, polyurethane and silicone foam; polyvinyl formal resin; and porous cellulose have been employed for cell colonization. However, the porous material favored extensively is polyurethane foam (Table 2.9).

In many cases including decolorization, immobilization of fungal mycelia has been shown to be useful more than free mycelia (Knapp *et al.*, 2001).

Table 2.9 Porous materials used in cell immobilization

Organisms	Supports[a]	Products
Microbial cells		
Botrycococcus braunii	PUF	Hydrocarbon
Clostridium acetoburylicum	NS	Aceton, butanol, ethanol
Escherichai coli	SF	Amylase
Thiobacillus ferroxidans	PUF	Ferrous iron oxidation
Saccharomyces cerevisiae	SS	Ethanol
Saccharomyces uvarum	PEF	Ethanol
Mucor ambiguus	PUF	γ-Linolenic acid
Penicillium chrysogenum	PUF	Penicillin
Phanerochaete chrysosporium	PUF	Lignin peroxidase
Rhyzopus arrhizus	PUF	Fumaric acid
Rhyzopus chinensis	PUF	Lipase
Trichoderma viride	SF	Cellulase
Mixed culture	PUF	Waste treatment
Plant cells		
Capsicum frutescens	PUF	Capsaicin
Humulus lupulus	PUF	Hop flavors
Animal cells		
Mouse myeloma	PVF	Immunoglubulin G
Hepatocyte	PVF	Culture
CHO-KI and Vero	PUF	Culture

Note: [a] NS – Natural sponge; PEF – polyester foam; PUF – polyurethane foam; PVF – polyviny formal resin; SF – Silicone foam; SS – Stainless steel.

Source: modified from Fukuda, 1995.

2.7.2 Immobilized cell bioreactor

Immobilized cell bioreactor can be divided into several different categories, including stirred tank bioreactors, fixed bed bioreactors, and fluidized bed bioreactors, according to flow pattern. The choice of bioreactor design for an immobilized cell process would depend on several factors, i.e., in aerobic cell, a sufficient supply of oxygen and removal of carbon dioxide generated by respiration will be needed.

The immobilization method and particle characteristics must also be considered. In stirred tank bioreactors, damage to particles through mechanical shearing is much greater than in fixed bed bioreactors. Thus, only relatively robust preparations of immobilized cells should be used in stirred tank bioreactors. Whereas preparations of immobilized cells on very small particles can result in an unacceptably high pressure drop and clogging problems when used in a fixed bed bioreactors, the use of a fluidized bed bioreactors can circumvent these problems (Fukuda, 1995).

There are several reports about immobilized fungal bioreactor used in decolorization purposes. Early designs were somewhat constrained by the dogma that agitation would result in low activity of LiP and, therefore, restricted decolorization. This influenced designs as researchers attempted to find ways of growing fungal biomass with out a high degree of agitation. Use was made of rotating biological contractor, packed bed bioreactor and fluidized bed bioreactor. There are few reports in which organisms are grown and used for decolorization in stirred tank bioreactors presumably because of concern about the effect of shear forces on enzymes or mycelial structure. Most authors prefer to use aeration to provide mixing as well as aeration.

2.7.3 Packed bed bioreactor

Packed bed bioreactors (Figure 2.14) are the one in a group of fixed bed bioreactors (e.g. plug flow bioreactor, trickling filter, rotating biological contractor; Webb, 1987) that cells are immobilized on large particles. These particles do not move with the liquid. There are three substrate flow possibilities in a packed bed: downward flow, upward flow, and recycling flow. Continuous packed bed bioreactors are the most widely used reactors for immobilized enzymes and immobilized microbial cells. In these systems,

it is necessary to consider the pressure drop across the packed bed or column, and the effect of the column dimensions on the reaction rate. For industrial applications, upward flow is generally preferred over downward flow because it does not compress the beds as downward flow does. When gas is produced during a reaction, upward flow is preferred. The recycling method is advantageous when the linear velocity of the substrate solution affects the reaction flow rate. This is because the recycling method allows the substrate solution to be passed through the column at a desired velocity.

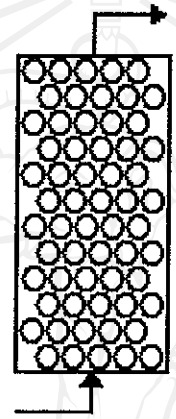

Figure 2.14 Upward flow packed bed bioreactor

Packed bed bioreactors are the most frequently employed type of immobilized cell bioreactor. The reactors have the advantages of simplicity of construction and operation and they can give a high reaction rate. However, they can suffer from blockages and from poor mass transfer.

The particulate catalysts, which are packed in such reactors, have high specific interfacial areas of solid-liquid contact, and the velocity of liquid creeping over the static solid particles substantially alleviates the film resistance of mass transfer. However, in the case of immobilized living cells, efficient gas-liquid contact and carbon dioxide removals are often necessary. Under this condition, a packed bed is often subject to accumulation of stagnant pocket of gas. This can cause gas flooding, which in turn produces poor liquid distribution and poor performance (Fukuda, 1995).

2.8 Related research

From the screening study of Jarosz-Wilkolaska *et al.* (2002), they collected several strain of fungi to test in decolorization of azo dye (Acid red 183) and anthraquinone dye (Basic blue 22) on agar medium. The results have been reported that *C. versicolor* can eliminate both two dyes within 8 and 5 day, respectively. While Knapp *et al.* (1995) studied in several dyes decolorized by many strain of *C. versicolor*. It was found that *C. versicolor* showed a high efficiency, 90-100%, in decolorization of azo dyes (Acid red 106, Mordant yellow 84, Brilliant yellow, Orange II), anthraquinone dye (Acid green 27), and other dye groups. For Wong and Yu (1999) and Wang and Yu (1998) reported that *C. versicolor* strain ATCC48424 and ATCC12679 could well decolorize azo dyes (Acid violet 7, Acid black 24), anthraquinone dyes (Acid green 27, Acid blue 25), and Indigo dye. Mechanisms of decolorization are biodegradation and biosorption. Laccase played a major role in biodegradation of these dyes, anthraquinone dyes were a substrate of laccase while azo and indigo dyes were degraded by action of laccase via small mediator, 8 kDa, produced as metabolite of the fungus.

C. versicolor RC3 is a white rot fungus isolated from Rukkachat park in Chiang Mai province. It could produce thermostable laccase (up to 55 $^{\circ}$C) and this extracted laccase could well decolorize anthraquinone dye (Remazol brilliant blue R) together with mediators and the culture of *C. versicolor* RC3 in dyes added basal medium (added with each of Orange II, Methyl orange, and Remazol brilliant blue R) could be decolorized completely within 3-4 day of incubation (Khanongnuch *et al.*, 2002).

For immobilization of *C. versicolor*, Pallerla and Chambers (1998) immbilized the fungus onto polyurethane foam and used in fluidized bed bioreactor for pentachlorophenol (PCB) degradation. The result showed that 20-25 mg/l of PCB could be degraded up to 99% when hydraulic retention time of the reactor was 12 hours. Another support, calcium alginate, used for immobilization of *C. versicolor* reported by Pallerla and Chambers (1997). They filled the immobilized beads into fluidized bed bioreactor and used this system for decolorization of paper mill effluent. It was found that about 70% of color removal occurred when 1 day of hydraulic retention time was used.

With packed bed bioreactor, there is no report about using of continuous immobilized packed bed bioreactor of *C. versicolor* in dye decolorization purpose. However, there are some reports of other fungi. *Irpex lacteus* was immobilized onto polyurethane foam and packed in a glass column for use in decolorization of Remazol brilliant blue R. It was found that this system could decolorized the dye 95-100% within 6-10 day and found more that decolorization was occurred by action of MnP (Kasinath *et al.*, 2003). While Mielgo *et al.* (2001) also immobilized *P. chrysosporium* on polyurethane foam and used the immobilized fungus in packed bed bioreactor for study of Orange II decolorization. The result showed that the reactor could remove color more than 95% when hydraulic retention time was 24 hours and MnP was reported that it played a major role in the degradation of Orange II.

There are few reports about by-product released in decolorization broth of Orange II. Dingenouts *et al.* (2000) was reported that 1,2-naphthoquinone (Figure 2.15) was released from Orange II degradation by the fungus *Pycnoporus cinnabarinus.*

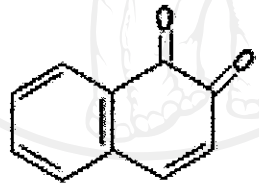

Figure 2.15 1,2-naphthoquinone