CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Textile industries consume large volumes of water and chemicals for wet processing of textile. The chemical reagents used are very diverse in chemical composition, ranging from inorganic compounds to polymers and organic products. The presence of very low concentrations of dyes in effluent is highly visible and undesirable (Nigam *et al*., 2000). There are more than 100,000 commercially available dyes with over $7x10^5$ ton of dyestuff produced annually. Due to their chemical structure, dyes are resistance to fading on exposure to light, water and many chemicals. Many dyes are difficult to decolorize due to their complex structure and synthetic origin. There are many structural varieties of dyes that fall into either the cationic, nonionic or anionic type. Anionic dyes are the direct, acid and reactive dyes. Brightly colored, water-soluble reactive and acid dyes are the most problematic, as they tend to pass through conventional treatment systems unaffected. Nonionic dyes refer to disperse dyes because they do not ionize in an aqueous medium. Concern arises as many dyes are made from know carcinogens such ad benzidine and other aromatic compounds.

The problem of color in textile dye house effluent and the possible problems associated with the discharge of dyes and dye degradation products are of concern. Traditional methods for dealing with this kind of wastewater are usually the biological, physical and chemical techniques as well as the various combinations of theses (Chen *et al*., 2005). It has been widely reported that many dye chemicals are difficult to degrade using conventional biological treatment processes.

2.2 Definition of Batik

Batik is the Indonesian word for wax resist dyeing. Liquid wax is applied to the fabric and the fabric is then dipped in the dye. The areas covered with wax resist the dye and retain their previous or original color. This waxing process is repeated with each new color. You begin by waxing the lightest areas of the design and proceed to dye with successively darker colors. Each color will affect the next color dyed over it.

Batik at the first may seem complicated and confusing. There are many types of fabric available, many choices of waxes, varieties of dyes, and, most confusing, numerous batiking procedures. But, if taken one step at a time, batik can be easily understood and the batiking procedure a joy of discovery (Hersk, 1975).

2.3 Classification of dyestuffs

Dyes are generally small molecules comprising two key components: the chromophore, responsible for the color, and the functional group, which bonds the dye to the fiber. Dyestuff is organic or inorganic and water-soluble substances which can absorb or reflect some lights to show color. The first man made organic dye, mauveine, was discovered by William Henry Perkin in 1856. Many thousands of dyes have since been prepared and because of vastly improved properties imparted upon the dyed materials quickly replaced the traditional natural dyes. Dyes are now classified according to how they are used in the dyeing process (Venceslau *et al*., 1994).

2.3.1 Acid dye

Acid dyes are water soluble anionic dyes that are applied to fibers such as silk, wool, nylon and modified acrylic fibers from neutral to acid dye baths. Attachment to

the fiber is attributed, at least partly, to salt formation between anionic groups in the dyes and cationic groups in the fiber. Acid dyes are not substantive to cellulosic fibers.

2.3.2 Basic dye

Basic dyes are water soluble cationic dyes that are mainly applied to acrylic fibers but find some use for wool, and silk. Usually acetic acid is added to the dye bath to help the take up of the dye onto the fiber. Basic dyes are also used in the coloration of paper.

2.3.3 Direct (Substantive) dye

Dyeing is normally carried out in a neutral or slightly alkaline dye bath, at or near the boil, with the addition of either sodium chloride (NaCl) or sodium sulfate (Na_2SO_4). Direct dyes are used on cotton, paper, leather, wool, silk and nylon. They are also used as pH indicators and biological stains.

2.3.4 Mordant dye

As the name suggests these dyes require a mordant. This improves the fastness of the dye on the fiber such as water, light and perspiration fastness. The choice of mordant is very important as different mordants can change the final color significantly. Most natural dyes are mordant dyes and there is therefore a large literature base describing dyeing techniques. The most important mordant dyes are the synthetic mordant dyes (chrome dyes) used for wool, these comprise some 30% of dyes used for wool and are especially useful for black and navy shades. The mordant used is potassium dichromate applied as an after-treatment.

2.3.5 Vat dye

These dyes are essentially insoluble in water and incapable of dyeing fibers directly. However, reduction in alkaline liquor produces the water soluble alkali metal salt of the dye. In this leuco form these dyes have an affinity for the textile fiber. Subsequent oxidation reforms the original insoluble dye.

2.3.6 Reactive dye

First appeared commercially in 1956 and were used to dye cellulosic fibers. The dyes contain a reactive group that, when applied to a fiber in a weakly alkaline dye bath, forms a chemical bond with the fiber. Reactive dyes can also be used to dye wool and nylon, in the latter case they are applied under weakly acidic conditions.

2.3.7 Disperse dye

They are substantially water-insoluble. The dyes are finely ground in the presence of a dispersing agent then sold as paste or spray dried and sold as a powder. They can also be used to dye nylon, triacetate, polyester and acrylic fibers. In some cases a dyeing temperature of $130^{O}C$ is required and a pressurized dye bath is used. The very fine particle size gives a large surface area that aids dissolution to allow uptake by the fiber. The dyeing rate can be significantly influenced by the choice of dispersing agent used during the grinding.

2.3.8 Azoic dye

A dyeing technique with an insoluble azo dye is produced directly onto or within the fiber. This is achieved by treating a fiber with a diazo component and a coupling component. With suitable adjustment of dye bath conditions, two components react to produce the required insoluble azo dye. This dyeing technique is unique in that the final color is controlled by the choice of the diazo and coupling components.

The Batik dyeing process usually uses 3 kinds of dye including direct dye, reactive dye and vat dye. The advantages and disadvantages of those were summarized in Table 2.1.

Table 2.1 The advantages and disadvantages of dyes use in Batik dyeing process

Dyes	Advantages	Disadvantages
Direct dye	Cheap and easy	Poor wet and light fastness
Reactive dye	High wet fastness and easy	Expensive
Vat dye	High wet fastness and easy	Expensive

Source: Venceslau *et al.*, 1994

2.4 Problems from Batik dyeing effluents and wastewater treatments

In Batik dyeing process, dyestuffs are lost in the industrial causing the problems as following:

2.4.1 Giving a high visibility to water bodies. The present of very small amounts of some dyes in water about 10-15 ppm is highly visible a visual pollution.

2.4.2 Be potentially converted to toxic and carcinogenic substances. Most of dyes used are synthetic dyes produced from know toxic and carcinogenic precursors such as benzidine 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. In recent years, there are more than 20 kinds of aromatic amines that can be potentially reduced and released from dyestuffs.

2.4.3 Reduce water transparency by their high visibility. This problem affect directly to photosynthetic organisms in aquatic ecosystems such as phytoplanktons and aquatic plants.

2.4.4 Complete to oxygen solubility when dissolved large amount in water causing the decreasing of dissolved oxygen which directly affect to aquatic lives.

When there are ant dyes contaminating into wastewater, several choices of dyes removal can be selected to solve the problems. The following paragraphs are classification of dye removal methods.

2.5 Methods of dye removal

2.5.1 Chemical treatments

2.5.1.1 Oxidative process

This is the most commonly used method of decolorization by chemical means. This is mainly due to its simplicity of application. The main oxidizing agent is usually hydrogen peroxide (H_2O_2). This agent needs to be activated by some means, for example, ultra violet light. Many methods of chemical decolorization vary depending on the way in which the H_2O_2 is activated (Slokar and Le marechal, 1997). Chemical oxidation removes the dye from the dye-containing effluent by oxidation resulting in aromatic ring cleavage if the dye molecules. Xu *et al*., (2005) decolorized 10 types of dye solutions by potassium permanganate. It was found that the decolorization rate of dye solutions by potassium permanganate was rapid, and most of dye solutions can be

decolorized effectively. The results of total organic carbon indicated that dye solutions were degraded incompletely. The results of treatment of textile wastewater indicated that the oxidation with potassium permanganate might be used as a pre-treatment process before biological treatment.

2.5.1.2 (H_2O_2-Fe(II) salts (Fenton reagent)

Fentons reagent is a suitable chemical means of treating wastewaters that are either resistant to biological treatment or poisonous to live biomass. Chemical separation used the action of sorption or bonding to remove dissolved dyes from wastewater and has been shown to be effective in decolorization both soluble and in soluble dyes (Pak and Chang, 1999). One major disadvantage of this method is sludge generation through the flocculation of the reagent and the dye molecules. The sludge, which contains the concentrated impurities, still requires disposal. It has conventionally been incinerated to produce power, but such disposal is seen by some to be far from environmentally friendly. The performance is dependent on the final floc formation and its settling quality, although cationic dyes do not coagulate at all. Acid, direct, vat mordant and reactive dyes usually coagulate, but the resulting floc is of poor quality and does not settle well, yielding mediocre results (Raghavacharya, 1997)

2.5.1.3 Ozonation

The use of ozone was first pioneered in the early 1970s, and it is a very good oxidizing agent due to its high instability compared to chlorine, another oxidizing agent and H_2O_2. Oxidation by ozone is capable of degrading chlorinated hydrocarbons, phenols, pesticides and aromatic hydrocarbons (Xu and Lebrun, 1999). The dosage applied to the dye-containing effluent is dependent on the total color and residual COD to be removed with no residue or sludge formation and no toxic metabolites. Ozonation leaves the effluent with no color and low COD suitable for discharge into

environmental waterways. This method shows a preference for double-bonded dye molecules. One major advantage is that ozone can be applied in its gaseous state and therefore does not increase the volume of wastewater and sludge.

Chromophore groups in the dyes are generally organic compounds with conjugated double bonds that can be broken down forming smaller molecules, resulting in reduced coloration (Peralto-Zamora *et al*., 1999). These smaller molecules may have increased carcinogenic or toxic properties, and so ozonation may be used alongside a physical method to prevent this. Decolorization occurs in a relatively short time.

A disadvantage of ozonation is its short half-life, typically being 20 min. This time can be further shortened if dyes are present, with stability being affected by the presence of salts, pH, and temperature. In alkaline conditions, ozone decomposition is accelerated, and so careful monitoring of the effluent pH is required. Better results can be achieved using irradiation or with a membrane filtration technique (Lopez *et al*., 1999). One of the major drawbacks with ozonation is cost, continuous ozonation is required due to its short half-line.

2.5.1.4 Photochemical

This method degrades dye molecules to CO_2 and H_2O by UV treatment in the presence of H_2O_2. Degradation is caused by the production of high concentrations of hydroxyl radicals. UV light may be used to activate chemicals, such as H_2O_2, and the rate of dye removal is influenced by the intensity of the UV radiation, pH, dye structure and the dye bath composition. This may be set-up in a batch or continuous column unit. Depending on initial materials and the extent of the decolorization treatment, additional by-products, such as, halides, metals, inorganic acids, organic aldehydes and organic acids, may be produced. There are advantages of photochemical treatment of dye-containing effluent; no sludge is produced and foul odors are greatly reduced.

2.5.1.5 Sodium hypochloride (NaOCl)

This method attacks at the amino group of the dye molecule by the Cl^+. It initiates and accelerates azo bond cleavage. This method is unsuitable for disperse dyes. An increase in decolorization is seen with an increase in chlorine concentration. The use of chlorine for dye removal is becoming less frequent due to the negative effects it has when release into waterway and the release of aromatic amines which are carcinogenic, or otherwise toxic molecules.

2.5.1.6 Cucurbituril

Cucurbituril was first mentioned by Behrand *et al.*, (1905), and the rediscovered in the 1980s by Freeman *et al.*, (1981). It is a cyclic polymer of glycoluril and formaldehyde. Cucuebituril, so named, because its structure is shaped like a pumpkin (a member of the plant family *Cucurbitaceae*). The uril, indicates that a urea monomer is also part of this compound. Cucurbituril is known to form host-guest complex with aromatic compounds and this may be the mechanism for reactive dye adsorption. Another proposed mechanism is based on hydrophobic interactions or the formation of insoluble cucurbituril dye-cation aggregates since adsorption occurs reasonably fast. To be incorporated into fixed bed sorption filters. Like many other chemical methods, cost is a major disadvantage.

2.5.1.7 Electrochemical destruction

This is a relatively new technique, which was developed in the mid 1990s. It has some significant advantages for use as an effective method for dye removal. There is little or no consumption of chemicals and no sludge build up. The breakdown metabolites are generally nit hazardous leaving it safe for treated wastewaters to be released back into waste ways. It shows efficient and economical removal of dyes and a high efficiency for color removal and degradation of recalcitrant pollutants.

Relatively high flow rates cause a direct decrease in dye removal, and the cost of electricity used is comparable to the price of chemicals.

2.5.2 Physical treatments

2.5.2.1 Adsorption

Adsorption techniques have gained favor recently due to their efficiency in the removal of pollutants too stable for conventional methods. Adsorption produces a high quality product, and is a process which is economically feasible. Decolorization is a result of two mechanisms: adsorption and ion exchange, and is influenced by many physio-chemical factors, such as, dye/sorbent interaction, sorbent surface area, particle size, temperature, pH, and contact time.

2.5.2.2 Activated carbon

This is the most commonly used method of dye removal by adsorption and is very effective for adsorbing cationic, mordant, and acid dyes and to a slightly lesser extent, dispersed, direct, vat, pigment and reactive dyes. Performance is dependent on the type of carbon used and the characteristics of the wastewater. Removal rates can be improved by using massive dosed, although regeneration or re-use results in a steep reduction in performance, and efficiency of dye removal becomes unpredictable and dependent on massive does of carbon. Activated carbon, like many other dye-removal treatments, is well suited for one particular waste system and ineffective in another. Activated carbon is expensive. The carbon also has to be reactivated otherwise disposal of the concentrates has to be considered. Reactivation results in 10-15% loss of the sorbent.

2.5.2.3 Peat

The cellular structure of peat makes it an ideal choice as an adsorbent. It has the ability to adsorb transition metals and polar organic compounds from dye-containing effluents. Peat may be seen as a viable adsorbent in countries such as Ireland and UK, where it is widely available. Peat requires no activation, unlike activated carbon, and also costs much less. Due to activated carbon's powdered nature, it has a much larger surface area, and hence has a better capacity for adsorption. Spent peat may be burned and utilized for steam rising, or, potentially, as substrate in solid state fermentation (SSF), for protein enrichment.

2.5.2.4 Wood chips

They show a good adsorption capacity for acid dyes although due to their hardness, it is not as good as other available sorbents and longer contact times are required. Adsorbed wood is conventionally burnt to generate power although there is potential for SSF of the dye-adsorbed wood chips.

2.5.2.5 Fly ash and coal (mixture)

A high fly ash concentration increases the adsorption rates of the mixture due to increasing the surface area available for adsorption. This combination may be substituted for activated carbon, with a ratio of fly ash:coal, 1:1

2.5.2.6 Silica gel

An effective material for removing basic dyes, although side reactions, such as air binding and air fouls with particulate matter, prevents it being used commercially.

2.5.2.7 Other materials

The use of these substrates such as natural clay, corn cobs, rice hull etc., for dye removal is advantageous mainly due to their widespread availability and cheapness. They are economically attractive for dye removal, compared to activated charcoal, with many comparing well in certain situations. These materials are so cheap regeneration is not necessary and the potential exists for dye-adsorbed materials to be used as substrates in SSF for protein enrichment.

Garg *et al*., (2004) used adsorbents prepares from *Prosopis cineraria* sawdust (an agro-industrial waste) to remove the malachite green from an aqueous solution in a batch reactor. The adsorbents included formaldehyde-treated sawdust (PCSD) and sulphuric acid-treated sawdust (PCSDC). The effects of adsorbent surface change, initial pH and dye concentration, adsorbent mass and contact time on dye removal have been determined. These experimental studies have indicated that PCSD and PCSDC could be employed as low-cost alternatives in wastewater treatment for the removal of dyes.

2.5.2.8 Membrane filtration

This method has the ability to clarify, concentrate and, most importantly, to separate dye continuously from effluent. It has some special features unrivalled by other methods; resistance to temperature, an adverse chemical environment, and microbial attack. The concentrated residue left after separation poses disposal problems, and high capital cost and the possibility of clogging, and membrane replacements are its disadvantages. This method of filtration is suitable for water recycling within a textile dyes, but it is unable to reduce the dissolved solid content, which makes water re-use a difficult task.

2.5.2.9 Ion exchange

Ion exchange has not been widely used for the treatment of dye-containing effluents, mainly due to the opinion that ion exchangers cannot accommodate a wide range of dyes. Wastewater is passed over the ion exchange resin until the available exchange sites are saturated. Both cation and anion dyes can be removed from dye-containing effluent this way. Advantages of this method include no loss of adsorbent on regeneration, reclamation of solvent after use and the removal of soluble dyes. A major disadvantage is cost. Organic solvents are expensive, and the ion exchange method is not very effective for disperse dyes.

2.5.2.10 Irradiation

Sufficient quantities of dissolved oxygen are required for organic substances to be broken down effectively by radiation. The dissolved oxygen is consumed very rapidly and so a constant and adequate supply is required. This has an effect on cost. Dye-containing effluent may be treated in a dual-tube bubbling rector. This method showed that some dyes and phenolic molecules can be oxidized effectively at a laboratory scale only.

2.5.2.11 Electrokinetic coagulation

This is an economically feasible method of dye removal. It involves the addition of ferrous sulphate and ferric chloride, allowing excellent removal of direct dyes from wastewaters. Unfortunately, poor results with acid dyes, with the high cost of the ferrous sulphate and ferric chloride, means that it is not a widely used method. The optimum coagulant concentration is dependent on the static charge of the dye in solution and difficulty in removing the sludge formed as part of the coagulation is a problem. Production of large amounts of sludge occurs, and this results in high disposal costs.

The advantages and disadvantages of some methods used in dye removal from industrial effluents were shown in Table 2.2.

Table 2.2 Advantages and disadvantages of the current methods of dye removal from industrial effluents

Physical/chemical methods	**Advantages**	**Disadvantages**
Fenton reagent (H_2O_2-Fe(II) salts)	Effective decolorization of both soluble and insoluble dyes	Sludge generation
Ozonation	Applied in gaseous state: no alteration of volume	Short half-life (20 min)
Photochemical	No slugged production	Formation of by-product
NaOCl	Initials and accelerates azo bond cleavage	Release of aromatic amines
Cucurbituril	Good sorption capacity for various dyes	High cost
Electrochemical destruction	Breakdown compounds are non-hazardous	High cost of electricity
Activated carbon	Good removal of wide variety of dyes	Very expensive
Peat	Good adsorbent due to cellular structure	Specific surface areas for adsorption are lower than activated carbon
Wood chips	Good sorption capacity for acid dyes	Requires long retention times
Silica gel	Effective for basic dye removal	Side reactions prevent commercial application

Table 2.2 (Continued)

Physical/chemical methods	Advantages	Disadvantages
Membrane filtration	Removes all dye types	Concentrated sludge production
Ion exchange	Regeneration: no adsorbent loss	Not effective for all dyes
Irradiation	Effective oxidation at lab scale	Requires a lot of dissolved oxygen
Electrokinetic coagulation	Economically feasible	High sludge production

Source: Robinson *et al*., 2001

2.5.3 Biological treatments

The biological decolorization processes are aerobic activated sludge or rotating biofilm reactors, aerobi-anaerobic packed-bed reactors, aerobic-anaerobic fluidized-bed reactors, aerobic-anaerobic sequential batch or continuous-flow reactors and anaerobic batch reactors (Banat *et al*., 1996). Kim *et al*. (2003) showed the effectiveness of biological pretreatment involving appropriate microorganisms and suitable support media in a combined process. The combined process consists of biological pretreatment, chemical coagulation and electrochemical oxidation. It was found that the combined process reduced COD and color 95.4% and 98.5%, respectively.

Shaw *et al*. (2002) developed a six-phase anaerobic/aerobic sequencing laboratory scale batch reactor to treat a synthetic textile effluent contained an azo dye (Remazol Black). The reactor removed 66% of the applied total organic carbon compared to 76% from a control reactor without dye. Color removal was 94% but dye

metabolites caused reactor instability. Aromatic amines from the anaerobic breakdown of the azo dyes were not completely mineralized by the aerobic phase.

2.5.3.1 Decolorization by microbial cultures

Mixed bacterial cultures from a wide variety of habitats have also been shown to decolorize the diazo linked chromophore of dye molecules in 15 days (Knapp and Newby, 1995). The advantages of mixed cultures are apparent as some microbial consortia can collectively carry out biodegradation tasks that no individual pure strain can undertake successfully. However, mixed cultures only provide an average macroscopic view of what is happening in the system and result are not easily reproduced, making through, effective interpretation difficult. For these reasons, a substantial amount of research on the subject of color removal has been carried out using single bacterial cultures. Nigam *et al*., (1996) and Nigam and Marchant (1995) demonstrated that a mixture of dyes were decolorized by anaerobic bacteria in 24-30 h, using free growing cells or in the form of biofilms on various support materials. Ogawa and Yatome (1990) also demonstrated the use of bacteria for azo dye biodegradation. These microbial systems have the drawback of requiring a fermentation process, and are therefore unable to cope with larger volumes of textile effluents.

The ability of bacteria to metabolize azo dyes has been investigated by a number of research groups. Under aerobic conditions azo dyes are not readily metabolized although Kulla (1981), reported the ability of *Pseudomonas* strains to aerobically degrade certain azo dyes. However, the intermediated formed by these degradative steps resulted in disruption of metabolic pathways and the dyes were not actually mineralized. Under anaerobic conditions, such as anoxic sediments many bacteria gratuitously reduce azo dyes reportedly by the activity of unspecific, soluble, cytoplasmic reductases, known as azo reductase. These enzymes are reported to result in the production of colorless aromatic amines which may be toxic, mutagenic, and possibly carcinogenic to animals.

Increasingly literature evidence suggests that additional processes may also be involved in azo dye reduction. It has been reported that many bacteria reduce a variety of sulfonated and non-sulfonated azo dyes under anaerobic conditions without specificity of any significance. In addition many highly charged and high molecular-sized sulfonated and polymeric azo dyes are unlikely which is not dependent on the intracellular availability of the azo dye (Keck *et al*., 1997). Pearce *et al*. (2003) reported that the simplest mechanism of color removal by whole bacterial cells is that of the adsorption of the dye onto the biomass. However, this mechanism is similar to many other physical adsorption mechanisms for the removal of color and is not suitable for long term treatment. This is because, during adsorption, the dye is concentrated onto the biomass, which will become saturated with time, and the dye-adsorbent composition must also be disposed. Bio-association between the dye and the bacterial cells tends to be the first step in the biological reduction of azo dyes, which is a destructive treatment technology.

Pala and Tokat (2002) studied the color removal from cotton textile industry wastewater in an activated sludge system with various additives. In this study, a specific organic flocculant (Marwichem DEC), powdered activated carbon (PAC), bentonite, activated clay and commercial synthetic inorganic clay (Macrosorb) were directly added into the activated sludge in laboratory pilot plant model. Before dosage, the optimum sludge retention time and hydraulic retention time were determined as 30 and 1.6 days, respectively. The most effective materials were found to be DEC and PAC for color removal. While the color removal efficiency for 120 mg/L DEC addition was 78%, it was 65% for 100 mg/L, 77% for 200 mg/L and 86% for 400 mg/L PAC addition.

Yeasts, such as *Klyveromyces marxianus*, are capable of decolorizing dyes. Banat *et al.* (1999) showed that *K. marxianus* was capable of decolorizing Remazol Black B by 78-98%. Zissi *et al*. (1996) showed that *Bacillus subtilis* could be used to break down p-amin-oazobenzene, a specific azo dye. Further research using mesophilic and thermophilic microbes has also shown them to degrade and decolorize dyes.

2.5.3.2 Adsorption by living/dead microbial biomass

The uptake or accumulation of chemicals by microbial mass has been termed biosorption. Dead bacteria, yeast, fungi and algae have all been used for the purpose of decolorizing dye-containing effluents. Table 2.3 and 2.4 show the onformation on the use of living and dead fungi to decolorize dyes, respectively (Fu and Viraraghavan, 2001).

Textile dyes vary greatly in their chemistries, and therefore their interactions with microorganisms depend on the chemistry of a particular dye and the specific chemistry of the microbial biomass. Depending on the dye and the specific microorganism used different binding rates and capacities will be observed. It can be said that certain dyes have a particular affinity for binding with microbial species.

It has been observed that biomass derived from the thermotolerant ethanol-producing yeast strain, *K. marxianus* IMB3, exhibited a relatively high affinity for heavy metals. Biosorption capacities showed that this type of biomass had a significantly high affinity for dye removal, and so widened the spectrum of use for biomass.

The use of biomass has its advantages, especially if the dye-containing effluent is very toxic. Biomass adsorption is effective when conditions are not always favorable for the growth and maintenance of the microbial population. Adsorption by biomass occurs by ion exchange.

Marungrueng and Pavasant (2006) used macroalga *Caulerpa lentillifera* to adsorb Astrazon Blue FGRL dye. The highest maximum adsorption capacity was obtained at 50 ^{0}C. The enthalpy of adsorption was estimated at 14.87 kJ mol^{-1} suggesting a chemical adsorption mechanism.

Hu (1992) demonstrated the ability of bacterial cells to adsorb reactive dyes. Zhou and Zimmerman (1993) used actinomyces as an adsorbent for decolorization of effluents containing anthroquinone, phalocyanine and azo dyes.

Biosorption tends to occur reasonably quickly: a few minutes in algae to a few hours in bacteria. This is likely to be due to an increase in surface area caused by cell rupture during autoclaving.

Table 2.3 Data on various living fungal cells capable of dye decolorization

Cultures	**Dye and concentration**	**Percent removal/ time**	**Mechanisms**
Aspergillus foetidus	Remazole Red (50 mg/l)	44	Biosorption
	Lignin (0.10%)	90 (2d)	Biosorption
A. niger	Basic Blue 9 (50%)	1.17* (2d)	Biosorption
Coriolus versicolor	Acid Green 27	100	Biodegradation and adsorption
Ganoderma sp.	Orange II (100 mg/l)	28-77 (2d)	Adsorption
Geotrichum fici	Reactive Black5	7*	Adsorption
Myrothecum verrucaria	Orange II (200mg/l)	70 (5h)	Adsorption
Phanerocheate chrysosporium	Indigo Caramine (40-50 mg/l)	29.8 (9d)	Ligninase-catalyzed
P. chrysosporium	Methyl Green (29µg)	88 (15 min)	Lignin peroxidase
Rhizopus oryzae	Reactive Blue 19	99*	Adsorption
Xeromyces bisporus	Sulfur Black 1 Dye waste	63*	Adsorption

Note: * mg of dye adsorbed/g of biomass

** initial decolorization rate (mg/l/h)

Source: Fu and Viraraghavan, 2001

Table 2.4 Data on various dead fungal cells capable of dye decolorization

Fungal strains	Dye and concentration	Biosorption capacity (mg of dye/g biomass)	Mechanism
Aspergillus niger	Basic Blue 9 (50mg/l)	10.49-18.54 (2d)**	Biosorption
A. niger	Reactive Brilliant Red (250 mg/l)	14.2 (2weeks)**	Adsorption
Botrytis cinerea	Reactive Blue 19 Dye waste	42	Adsorption
Fomitopsis carnea	Basic Violet 16 (50 mg/g)	503.1	Adsorption
Geotrichum fici	Reactive Black 5 Dye waste	45	Adsorption
Phanerochaete chrysosporium	Congi Red (500 mg/l)	90* (2d)** (liquid culture)	Adsorption
Rhizopus oryzae (26668)	Reactive Brilliant Red (250 mg/l)	102.6 (4 weeks)**	Adsorption
Xeromyces bisporus	Reactive Blue 19 Dye waste	60	Adsorption

Note: * % color removal

** numbers within brackets relate to time of contact between the dye and the adsorbent

Source: Fu and Viraraghavan, 2001

2.5.3.3 Anaerobic textile-dye bioremediation systems

Azo dyes make up 60-70% of all textile dyestuffs. Azo dyes are soluble in solution, and are not removed via conventional biological treatments. Reactive dyes have been identified as the most problematic compounds in textile dye effluents.

Anaerobic bioremediation allows azo and other water-soluble dyes to be decolorized. This decolorization involves an oxidation-reduction reaction with hydrogen rather than free molecular oxygen in aerobic systems. Typically, anaerobic breakdown yields methane and hydrogen sulphide.

Azo dye acts as an oxidizing agent for the reduced flavin nucleotides of the microbial electron chain and is reduced and decolorized concurrently with reoxidation of the reduced flavin nucleotides. In order for this to occur, additional carbon is required in order for decolorization to proceed at a viable rate. This additional carbon is converted to methane and carbon dioxide, releasing electrons. These electrons cascade down the electron transport chain to a final electron acceptor, in this case, the azo-reactive dye. The electrons react with the dye reducing the azo bonds, and ultimately causing decolorization.

In lab-scale studies glucose has been added to provide a source of carbon. This additional carbon supplementation may be a limiting factor when this technology is scale-up. Anaerobic degradation of textile dye yields only azo reduction. Mineralization does not occur. It has been shown that azo and nitro components are reduced in the sediments and in the intestinal environment, resulting in the regeneration of the parent toxic amines. Therefore, careful monitoring is required before treated wastewater is released into waterways.

A major advantage of this anaerobic system, apart from the decolorization of soluble dyes, is the production of biogas. Biogas can be reused to provide heat and power, and will reduce energy costs.

Most of synthetic dyes are removed slowly by the biological treatment processes, because of their toxicity to indigenous microorganisms. Dye removal from wastewater by established biological treatment plant processes are expensive and need careful application. Furthermore, with the following anaerobic digestion, nitrogen-containing dyes are transformed into aromatic amines that are more toxic and mutagenic than the parental molecules (Lacina *et al*., 2003). To overcome these difficulties, various fungi are being investigated for their potential to decolorize effluents and the most widely studied are white rot fungi.

2.6 White rot fungi

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 2.1).

Figure 2.1 White area of wood degraded by white rot fungi (*Coriolus versicolor*)
Source: http://users.quista.net/sjgall/Brackets.htm

2.6.1 Application in bioremediation

White rot fungi have the ability to degrade many xenobiotic compounds with a wide variety of structures. The potential for use 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 2.5).

Table 2.5 Environmental pollutants degraded by white rot fungi

Types	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 such as *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*, *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*, and *Trametes (Coriolus) versicolor*, *T. hirsuta* and *Trametes* spp. (Wesenberg *et al*., 2003; and Knapp *et al.*, 2001). 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.

2.6.2 *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* is 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 shows an important role to degrade lignin in the wood to obtain nitrogen for surviving. *C. versicolor* seems 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; lignin peroxidase, manganese peroxidase, 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).

Coriolus versicolor RC3 (Figure 2.2) is a thermotolerant basidiomycete isolated from Chiang Mai province. Kitwechkun, (2004) used *C. versicolor* RC3 decolorized Orange II dye in continuous packed bed bioreactor and was found that 90% of decolorization was maintained over 120 hours.

Figure 2.2 *Coriolus versicolor* strain RC3 fruiting bodies (left), spores (middle) and colony (right)
Source: Kakumyan, 2005

2.7 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 2.6 show various ligninolytic enzyme secretion 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).

Table 2.6 Various ligninolytic enzymes secretion by white rot fungi

Species	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

2.7.1 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 2.3 show 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).

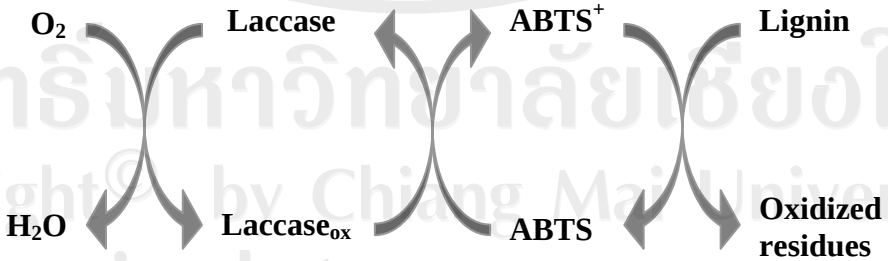

Figure 2.3 Catalytic cycle of laccase

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

2.7.2 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.

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

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

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

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

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 2.4.

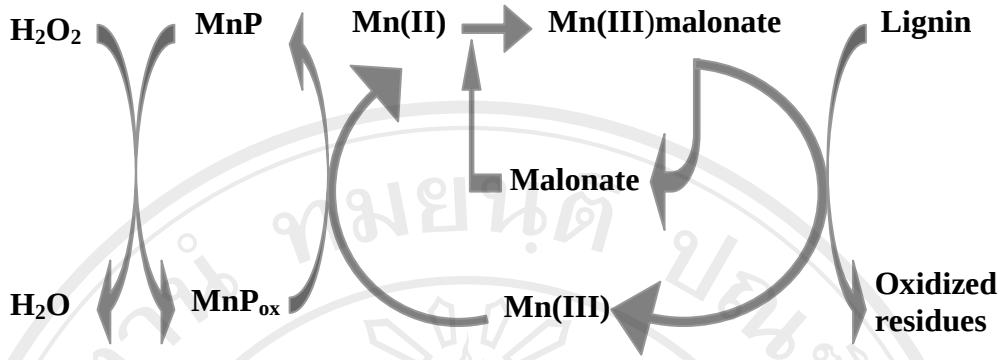

Figure 2.4 Catalytic cycle of manganese peroxidases
Source: modified from Archibald *et al*., 1997.

2.7.3 Lignin peroxidase

Lignin peroxidase (LiP, 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 2.5). VA is a fungal metabolite produced at the same time as LiP (Harvey and Thurston, 2001).

Young and Yu (1997) studied the decolorization of eight synthetic dyes including azo, anthraquinone, metal complex and indigo in white rot fungal cultures and by fungal peroxidase-catalysed oxidation. Dye decolorization rate increased

linearly with ligninase dosed. It was indicated that a highly efficient bioprocess using white rot fungi to romove color from industrial effluents should produce ligninase, H_2O_2, veratryl alcohol continuously and coordinately under acidic condition and controlled back-mixing flow of wastewater.

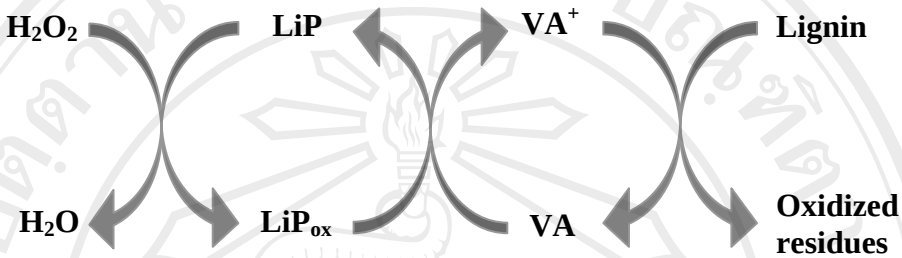

Figure 2.5 Catalytic cycle of lignin peroxidases
Source: modified from Harvey and Thurston, 2001.

2.7.4 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 2.7. 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.

Table 2.7 Some native and synthetic mediators in ligninolytic enzyme systems

Mediators	Enzymes	Organisms
Native mediators		
Mn(III)	MnP	*P. chrysosporium*
Organic acids (malonate, oxalate)	LiP, MnP	*P. chrysosporium, P. ostreatus, P. radiata*
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*
Violuric acid	Laccase	*C. versicolor, P. cinnabarinus,*
ABTS	Laccase	*C. versicolor, P. ostreatus*

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

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).

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

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

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

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

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

$$COOH–CHO + Mn(III) + ½O_2 \rightarrow HCOOH + CO_2^{*-} + Mn(II) \quad (2.10)$$

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

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

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

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

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 complexes 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 2.6) 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).

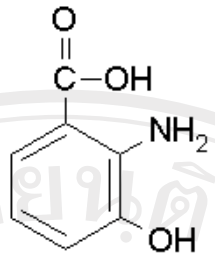

Figure 2.6 3-Hydroxyanthranilic acid
Source: Wesenberg *et al*., 2003

2.7.5 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 has been reported.

2.7.6 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).

2.7.7 Applications

In the same way of white rot fungi, application of their ligninolytic enzymes in waste treatment are similarity and have been reviewed in 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 2.8.

Rodriguez *et al*. (1999) reported that laccase, manganese peroxidase, lignin peroxidase, and aryl alcohol oxidase activities were determined in crude extracts from solid-state cultures of 16 different fungal strains grown on whole oats. All *Pleurotus ostreatus* strains exhibited high laccase and manganese peroxidase activity, but highest laccase volumetric activity was found in *Trametes hispida*. Solid-state culture on whole oats showed higher laccase and manganese peroxidase activities compared with growth in a complex liquid medium.

Table 2.8 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

2.8 Decolorization by white rot fungi

2.8.1 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 2.9.

Table 2.9 Decolorization of 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 radiate	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 bad reactor, hollow fibre reactor
	Phlebia radiate	Culture

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

2.8.2 Dye decolorization mechanisms

Fungal decolorization can be classified into two kinds, living cells to biodegradation by their ligninolytic enzymes and biosorption dyes to their biomass.

2.8.2.1 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.

For *C. versicolor*, dye decolorization was dependent on dye structures. Anthraquinone dye was laccase substrates directly while azo and indigoid dyes were not the substrates of laccase (Wang and Yu, 1998). Pathway of indigo dye degradation by laccase have been demonstrated by Campos *et al*. (2001) that laccases used in their experiment was produced and purified from *Trametes hirsuta* and *Sclerotium rolfsii*.

Kitwechkun and Khanongnuch (2004) studied the decolorization of azo dye (Orange II) by immobilized white rot fungus *C. versicolor* RC3. Physiological effect of some nutrients was investigated in batch experiment; ammonium oxalate was used as nitrogen source. It was found that the optimal concentration of ammonium oxalate was 0.2 g/l inducinh 97-98% decolorization after incubated on 120 rpm rotary shaker for 60-75 h.

Martin *et al*. (2003) screened several fungi for degradation of syringol derivatives of azo dyes possessing either carboxylic or sulphonic group. *T. versicolor* showed the best biodegradation performance and its potential was confirmed by the degradation of differently substituted fungal bioaccessible dyes. Biodegradation assays using mixtures of these bioaccessible dyes were performed to evaluate the possibility of a fungal wastewater treatment for textile industries.

Abadulia *et al*. (2000) reported that *T. hirsute* and a purified laccase from this organism was able to degrade triarylmethane, indigoid, azo, and anthraquinonic dyes. Immobilization of the *T. hirsute* laccase on alumina enhanced the thermal stabilities of the enzyme and its tolerance against some enzyme inhibitors, such as halides, copper chelators, and dyeing additives. Treatment of dyes with the immobilized laccase reduced their toxicities by up to 80% (anthraquinonic dyes).

2.8.2.2 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 depends 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 occur (Wang and Yu, 1998).

Bayramoglu *et al*. (2003) used immobilized and heat inactivated *T. versicolor* mycelia in carboxymethylcellulose, CMC for the biosorption of Cu^{2+}, Pb^{2+} and Zn^{2+} ions. The maximum biosorption capacities for both immobilized and heat inactivated *T. versicolor* were 1.51 and 1.84 mmol Cu^{2+}, 0.85 and 1.11 mmol Pb^{2+} and 1.33 and 1.67 mmol Zn^{2+} per g of dry biosorbents, respectively.

Arica *et al*. (2000) studied the biosorption of cadmium ions onto entrapped *T. versicolor* mycelia in Ca-alginate beads in batch system. The maximum experimental biosorption capacities for entrapped live and dead fungal mycelia of *T. versicolor* were found as 102.3 mg Cd (II) g^{-1} and 120 mg Cd (II) g^{-1}, respectively. The biosorbents were reused in three consecutive adsorption/desorption cycles without a significant loss in the biosorption capacity.

2.8.3 Dye decolorization using bioreactors

Kasinath *et al*. (2003) used immobilized white rot fungus *Irpex lacteus* on polyurethane foam and pine wood to study the effect of growth conditions on degradation of commercial dyes. It was found that both immobilized cultures were able to rapidly decolorization not only RBBR but also various textile industry and color bath effluent. Reusability and regenerative capacity of the immobilized cultures, important for application to water bioremediation, were documented.

Fujita *et al*. (2000) developed a bench-scale bioreactor using immobilized fungal equipped with an ultramembrane filtration unit to decolorize brow color component (melanoidins) arising from the heat-treatment liquor (HTL) of waste sludge. Artificial HTL containing 4200 color units of synthetic melanoidin supplemented with 100 mg/l ethanol was first subjected to decolorization by the *Coriolus hirsutus* IFO4917 immobilized onto polyurethane foam cubes. Then, the resultant biologically treated HTL was subjected to ultrafiltration to obtain the filtrate

as the effluent. It was found that a hydraulic retention time of 2 d in a 1d cycle sequencing batch mode, about 70% decolorization was routinely achieved using the entire systems (bioreactor+ultrafiltration), while the contribution of the fungal bioreactor alone to the decolorization was about 45%.

Zhang *et al*. (1999) reported that the reactors systems used in dye decolorization by white rot fungi included stirred tank reactors, a packed-bed bioreactor, airlift reactors or bubble columns, a rotating disc reactor and silicone membrane reactors, etc. In addition, they used the three different reactor configuration (continuous packed-bed bioreactor, fed batch fluidized-bed bioreactor and continuous fluidized-bed bioreactor) to design and test for decolorization of an azo dye, Orange II, with white rot fungus. It was found that the fed batch fluidized-bed bioreactor was particularly suitable for Orange II decolorization since it showed very high decolorization efficiency (over 97% color removal of 1000 mg/l Orange II in one day with immobilized fungal mycelium).

2.9 Toxicity test

Toxicity tests are desirable in water quality evaluations because chemical and physical tests are not sufficient to assess potential effects on aquatic biota (Grothe *et al*., 1996). For example, the effects of chemical interactions and the influence of complex matrices on toxicity cannot be determined from chemical tests alone. Different species of aquatic organisms are not equally susceptible throughout the life cycle. Even previous exposure to toxicants can alter susceptibility. In addition, organisms of same species can respond differently to the same level of a toxicant from time to time, even when all other variables are held constant.

Toxicity tests are useful for a variety of purposes that include determining as follow:

1. suitability of environmental conditions for aquatic life
2. favorable and unfavorable environmental factors, such as dissolved oxygen (DO), pH, temperature, salinity, or turbidity

3. effect of environmental factors on waste toxicity
4. toxicity of wastes to a test species
5. relative sensitivity of aquatic organisms to an effluent or toxicant
6. amount and type of waste treatment needed to meet water pollution control requirements
7. effectiveness of waste treatment methods
8. permissible effluent discharge rates
9. compliance with water quality standards, effluent requirements, and discharge permits

In such regulatory assessments, use toxicity test data in conjunction with receiving-water and site-specific discharge data on volumes, dilution rates, and exposure times and concentrations.

2.9.1 Types of toxicity tests: their uses, advantages and disadvantages

Toxicity tests are classified according to (a) duration: short-term, intermediate, and/or long-term, (b) method of adding test solutions: static, renewal, or flow-through, and (c) purpose: effluent quality monitoring, single compound testing, relative toxicity, relative sensitivity, taste or odor, or growth rate.

Short-term toxicity tests are used for routine monitoring suitable for effluent discharge permit requirements and for exploratory tests. They may use end points other than mortality as an end point or other discrete observations to determine effects due to the toxicant. These tests also may be used to indicate a suitable range of toxicant concentrations for intermediate and long-term tests. Short-term tests, rather than longer-duration tests, are used to obtain toxicity data as rapidly and inexpensively as possible. They are valuable for estimation of overall toxicity, for screening test solutions or materials for which toxicity data do not exist, for assessing relative toxicity of different toxicants or wastes to selected test organisms, or for relative sensitivity of different organisms to different conditions of such variables as temperature and pH. The results of these tests can be used to calculate acceptable

concentrations for very short exposures, such as those that might occur as organisms pass through an effluent zone of initial dilution or a mixing zone.

Toxicity tests of intermediate duration typically are used when longer exposure durations are necessary to determine the effect of the toxicant on various life stages of long-life-cycling organisms, and to indicate toxicant concentrations for life-cycle tests.

Long-term toxicity tests are generally used for estimating chronic toxicity. Long-term testing may include early-life-stage, partial-life-cycle, or full-life-cycle testing. Exposures may be as short as 7 days to expose specific portions of an organism's life cycle, 21 to 28 days to several months or longer for traditional partial-life-cycle and full-life-cycle tests with fish.

2.9.2 Selecting test organisms

The prime considerations in selecting test organisms are: their sensitivity to the factors under consideration; their geographical distribution, abundance, and availability within a practical size range throughout the year; their recreational, economic, and ecological importance and relevance to the purpose of the study; their abiotic requirements and whether these requirements approach the conditions normally found at the study site; the availability of culture methods for rearing them in the laboratory and a knowledge of their physiological and nutritional requirements; and their general physical condition and freedom from parasites and disease. To select a best species consider available information on sensitivity, consult with local authorities in pollution control or fish and wildlife agencies, or determine sensitivity with short-term tests. Select the test species bases on the considerations lists as well as organism size and life-cycle length. For testing of early life stages of organisms, species having a short life cycle are most cost-effective, but some tests require larger organisms with long life cycles.

For studies to determine effluent effects, select species representative in the area impacted. In most cases, use of laboratory cultured species is preferable to use of

those collected from the field. Laboratory-cultured organisms, either from in-house cultures or purchased from commercial bioassay organism suppliers, are of known age and quality. This allows for use of the most sensitive life stages throughout the year. Their use also may be more cost effective and allows for better quality assurance and control. For each series of tests, use organisms from a single source. Choose organisms that are nearly uniform in size, and for fish, with the largest individual not more than 50% longer than the shortest. Use organisms of the same age group or life stage. Optimally, conduct reference toxicant tests on cultured shocks and on lots of acquired or collected organisms. Knowledge of their environmental requirements and food habitats is important in selecting test organisms. Methods for laboratory holding and culturing are well described for a number of standard test species. When the purpose of the testing is site-specific, it may be necessary to collect certain life stages of selected organisms from the field for testing.

2.9.3 *Daphnia*

Daphnia (or Daphnids) are members of a collection of animals that are broadly termed as "water fleas". These are predominantly small crustaceans, and *Daphnia* belong to a group known as the Daphniidae. These organisms get their common name from their jerky movement through the water. Apart from the jerky movements, the resemblance to real fleas (*Pulex iritans*, etc), and ends: real fleas are insects and share only an extremely distant common ancestry with *Daphnia*, since both crustaceans and insects are arthropods (Figure 2.7). *Daphnia* sp. is small freshwater crustaceans that play an important role in aquatic ecology as an important source of food for fish and other aquatic organisms. *Daphnia* are excellent organisms to use in bioassays because they are sensitive to the changes in water chemistry and are simple and inexpensive to rise in an aquarium. *Daphnia* sp. is capable of fast maturity just within a few days, so it does not take long to grow a culture of test organisms. They have been used for many years to assess the acute and chronic effects of single chemicals and complex mixtures.

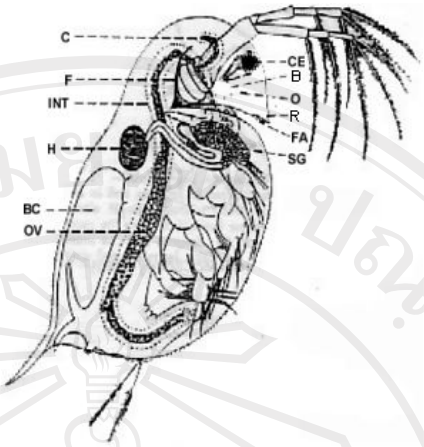

Figure 2.7 Anatomy of female *Daphnia pulex*; diagrammatic; *B*, brain; *BC*, brood chamber; *C*, digestive caecum; *CE*, compound eye; *F*, fornix; *FA*, first antenna (antennule); *H*, heart; *INT*, intestine; *O*, ocellus; *OV*, ovary; *R*, rostrum or beak; *SG*, shell gland.

Source: http://www.caudata.org

Because *Daphnia* are transparent, it is possible to conduct bioassays using endpoints other than death. For example, through a microscope, heart rate can be observed whether they have been eating. (Both of these signs are used to measure stress). Villegas-Navarro *et al*. (2001) used daphnids as bioassays to determine the LC50 values of textile wastewater sample taken from different stages of the finishing textile industries. All effluents from the five company samples were toxic in terms of LC50 and exhibited very high toxicity with acute toxicity unit (ATU) levels between 2.2 and 960, indicating that the five textile industries produced toxic water. Villarroel *et al*. (2003) tested acute and chronic toxicity of propanil on *Daphnia magma* using sublethal propanil concentrations during 21 days and the effect on survival, reproduction and growth of *D. magma* organism was monitored. It was found that feeding rates of *D. magma* declined with increasing propanil concentrations.