STUDIES ON THE DEGRADATION OF NITROPHENYLAZOPHENYLAMINE DYES BY THE WHITE ROT FUNGUS *PLEUROTUS OSTREATUS*

by

WANG LU

(Under the Direction of Ian R. Hardin)

ABSTRACT

Dyes are a group of chemicals that have been widely used in various industries, including textile industry. More than half of dyes have a nitrogen-nitrogen double bond structure in their molecules. These dyes are called azo dyes. Research on removing dyes from waster water has been done for decades. Recently, biodegradation using white rot fungi has emerged as an alternative method to traditional physical and chemical means for degradation of the dyes. Even though extensive research has been done to study the degradation of dyes by white rot fungi, the specific mechanism of such degradations, and how factors such as pH, buffer, and temperature can influence the degradation are still not fully understood. This study investigated the influence of buffer concentration, pH, temperature and agitation on fungal growth and fungus' ability to decolorize three water soluble dyes. The optimal culture parameters were established and then applied to degradations of five azo dyes. The degradation products were identified by Gas chromatography/mass spectrometry (GC/MS) and High performance liquid chromatography (HPLC). Pathway of the degradations of both dyes and their primary products were proposed.

INDEX WORDS: Azo dyes, White rot fungi, Biodegradation, Buffer, pH, Temperature, Agitation, Identification, Gas chromatography/mass spectrometry, High performance liquid chromatography, Pathway

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by

WANG LU

B.E. DongHua University (former China Textile University), China, 1998

M.E. DongHua University (former China Textile University), China, 2001

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WANG LU

Major Professor: Ian R. Hardin

Committee: Charles Yang
I. Jonathan Amster
Karen K. Leonas
Willam S. York

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

To my parents and my wife, for their love and patience in me.
To the memory of Jingzhe.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

The first recorded use of dye can be traced back to 2600 BC in China. In 1856, more than 4400 years later, William Henry Perkin accidentally discovered the first synthetic dye "Mauve" while trying to create synthetic quinine. Since then, thousands of dyes have been manufactured and applied in various industries, such as textile, food and automotive. Nowadays, India, the former USSR, Eastern Europe, China, South Korea and Taiwan consume approximately 600,000 tons of dyes annually (Ishikawa et al., 2000).

Depending on the molecular structure, dyes can often be classified into categories as azo, anthraquinone, and phthalocyanine. Among all known dyes, more than half contain one or more azo bonds in their molecular structures. These dyes are considered as “azo dyes”. Azo dyes normally have high colorfastness and encompass the entire visible spectrum. Many of them are easily synthesized from inexpensive and easily obtained starting materials (Bumpus, 1995).

Dyes are designed and synthesized to have affinity to the materials to be dyed and resistance to fading in end uses. Thus most dyes are quite difficult to decolorize or degrade when they are discharged into the environment from sources such as textile mills. In the textile industry, up to 15 percent of used dyes are released to processing water, which can give rise to not only visible problems but also environmental concerns (Vaidya and Datye, 1982). Many countries have established laws and regulations to reduce the environmental problems brought about by waste textile effluents (Robinson, T. et al., 2001). Such laws and regulations are

increasingly being enforced by the governments since some research has reported that the environmental transformation of a few dyes can generate potential carcinogenic and mutagenic compounds such as aromatic amines, and nitro-aromatic compounds (Bumpus, 1995, Baughman and Perenich, 1988, Weber and Wolfe 1987, Chung et al., 1978). Different physical and chemical methods have been developed to remove the dyes in textile waste effluents in past decades. However, these methods all have their disadvantages (Table 1.1). Research is still ongoing to find less expensive, more effective and environmentally friendly alternatives (Robinson, T. et al., 2001).

Over the last two decades, extensive research has been done on using biological techniques to decolorize and degrade colorants in wastewater. Many microorganisms, mainly bacteria and fungi, have shown the ability to decolorize dyes. In the case of bacteria, the degradation often involves an anaerobic reductive cleavage of the azo bond, and this can happen either extracelluarly or intracelluarly (Chung et al., 1993; Mechsner et al. 1982). Only a few bacteria species have been found to decolorize dye aerobically (Jiang and Bishop, 1994). Systems have been established to degrade azo dyes in wastewater to achieve complete mineralization. In such systems, azo dyes are first reduced anaerobically, followed by subsequent aerobic treatment to further degrade the dye to carbon dioxide and water (Seshadri et al., 1994; Bumpus 1995).

Other than bacteria, white rot fungi (WRF) are believed to be the most efficient single class of microorganism in breaking down synthetic dyes because of their lack of substrate specificity and capability of degrading a wide range of xenobiotics (Wesenberg et al., 2003). There have been many studies using WRF to decolorize wastewater over the past 20 years. Most of the attention has been paid to the a white rot fungus named *Phanerochaete chrysosporium*

(Chao and Lee et al., 1994; Cripps et al., 1990; Spadaro et al., 1992 and 1994; Ollikka et al., 1993), from which Tien and Kirk made their milestone discovery of two ligninolytic

Table 1.1 Advantages and disadvantages of the current methods of dye removal from industrial effluents (Robinson, T. et al., 2001)

Physical/chemical methods	Advantages	Disadvantages
Fentons reagent	Effective decolorization of both soluble and insoluble dyes	Sludge generation
Ozonation	Applied in the gaseous state: no alteration of volume	Short half-life (20 min)
Photochemical	No sludge production	Formation of by-products
NaOCl	Initiates 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 a 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
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 O2
Electrokinetic coagulation	Economically feasible	High sludge production

peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP) (Tien and Kirk, 1983). Besides *Phanerochaete chrysosporium,* other fungi such as *Pleurotus ostreatus, Trametes versicolor, Bjerkandera* and *Irpex lacteus* have also been recognized for their decolorizing ability (Heinfling et al., 1997 and 1998a; Martins et al., 2003; Novotny et al., 2001; Shin and Kim, 1998). In spite of the findings which demonstrated that WRF could decolorize a variety of dyes, questions still remain as to whether such dye molecules are eventually degraded to CO_2 and mineralized. The few studies that addressed this question showed that several ^{14}C-labelled azo dyes were indeed mineralized to $^{14}CO_2$ in the presence of WRF (Paszczynski et al., 1992, Sparado et al., 1992).

1.1 White rot fungi and their enzymes

Wood-rotting basidiomyceteous fungi which cause white-rot in wood are the most efficient lignin degraders in the nature. Biodegradation of lignin has been studied extensively since the 1970's. In the 1980's, studies predominantly focused on the biochemistry of lignin-degrading enzymes. Three major extracellular enzymes are known to be produced by various WRF. They are LiP (lignin peroxidase), MnP (manganese peroxidase), and laccase. Some WRF produce all three enzymes while others produce only one or two of them (Hatakka, 1994). These enzymes are produced by WRF during their secondary metabolism since lignin oxidation provides no net energy to fungi, and the synthesis and secretion of these enzymes are often induced by limited nutrient levels (mostly C or N) (Wesenberg et al., 2003).

Lignin peroxidase (LiP) is a type of heme-containing glycoprotein which requires hydrogen peroxide as an oxidant. LiP catalyzes the oxidation of nonphenolic aromatic lignin and similar compounds (Hatakka, 2003). Catalytic oxidation of side chains of lignin has been

reported (Tien and Kirk., 1983), as well as cleavage of aromatic ring structures (Umezawa and Higuchi, 1987). The catalytic cycle of LiP is illustrated in Figure 1.1 (Wesenberg et al., 2003). Such a mechanism can also apply to MnP. Native peroxidase is first oxidized by H_2O_2 to form compound I. The H_2O_2 is often produced by glyoxal oxidase and superoxide dismutase intracellularly (Leonowicz et al., 1999). Compound I then accepts one electron offered by suitable substrates to become compound II. Compound II is finally reduced back to native peroxidase by the substrates to complete the catalytic cycle, or be further oxidized to form compound III, which is regarded as an irreversible inactivation (Wesenberg et al, 2003). Substrates consumed by LiP could be lignin as well as recalcitrant xenobiotics such as textile dyes (Wesenberg et al, 2003; Zheng and Obbard, 2002, Valli et al., 1992). A model substrate for LiP is veratryl alcohol (VA), which is also secreted by the fungi themselves. The specific role played by VA has been studied by a number of researchers. It is suggested that VA is oxidized by LiP to form a cation radical, and that this radical then acts as a diffusible oxidant, mediating the oxidation of compounds that are inaccessible to the LiP active site (Harvey et al., 1986). Others believe that VA's function is merely to protect LiP from the inactivation by H_2O_2 (Valli et al. 1990). Later work showed that VA does act as mediator for certain substrates such as the phenolic compound, guaiacol (Koduri and Tien, 1995).

Manganese peroxidase (MnP), another type of heme-containing glycoprotein, also plays a significant role in lignin degradation. MnP preferentially oxidizes Mn^{2+} to Mn^{3+}, the latter then forming chelates with organic acids such as oxalic acid, which are also excreted by the fungi (Kuan and Tien, 1993; Wariishi et al., 1992). Such chelates can act as highly reactive redox-mediators to oxidize phenolic compounds (Hatakka 1994). It has to be recognized that

compound I of MnP can be reduced by both Mn^{2+} and phenolic compounds, whereas only Mn^{2+} can reduce compound II to native peroxidase (Wariishi et al 1988).

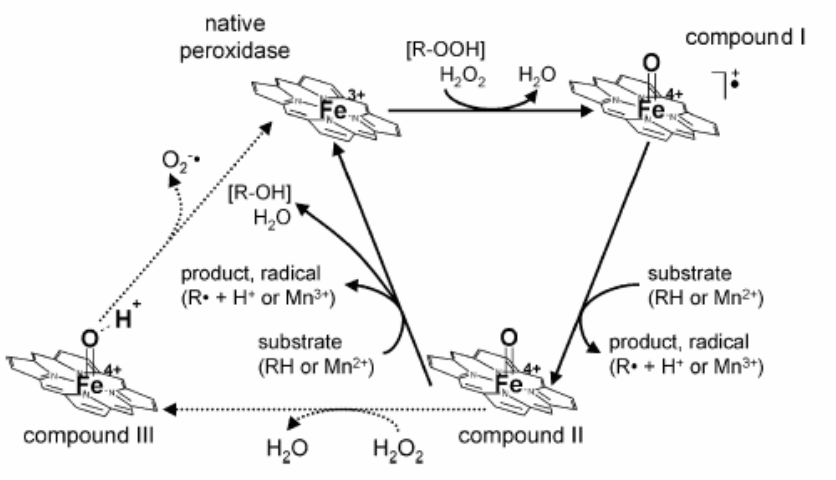


Figure 1.1 Generic scheme of the catalytic cycles of peroxidases (Wesenberg et al., 2003).

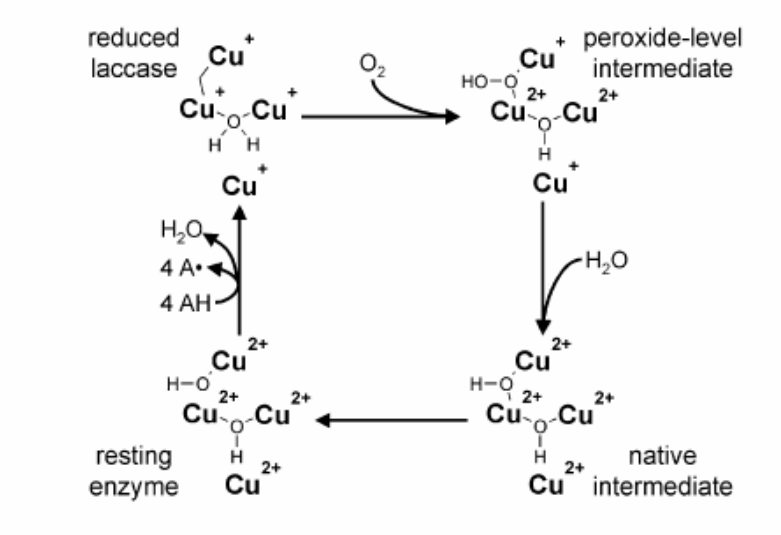


Figure 1.2 Illustration of the catalytic cycle of laccases (Wesenberg et al., 2003).

Laccase is a group of N-glycosylated extracellular blue oxidases produced by some WRF. Laccases contain four copper atoms in the active site (Call and Mücke, 1997) and catalyze the oxidation of a variety of aromatic hydrogen donors with the concomitant reduction of oxygen to water (Figure 1.2, Wesenberg, D. 2003). Moreover, laccases not only oxidize phenolic and methoxy-phenolic acids, but also decarboxylate them and attack their methoxy groups (Wesenberg et al., 2003)

In addition to LiP, MnP and laccase, another group of peroxidases, versatile peroxidases (VP), has been reported to be produced by WRF. Versatile peroxidase (VP) can be regarded as a hybrid between LiP and MnP, since they can oxidize not only Mn^{2+} but also phenolic and nonphenolic aromatic compounds, including dyes. This group of peroxidases has been found from species of *Pleurotus* and *Bjerkendera* (Heinfling et al., 1998a and 1998b; Mester and Field, 1998).

1.2 Decolorization or degradation of textile dyes by white rot fungi

Due to the non-specificity of WRF and the structural similarity between dyes and lignin, many attempts have been made to use WRF to decolorize dyes. Early efforts have concentrated on using *Phanerochaete chrysosporium* to decolorize various dyes due to its much studied degradation of lignin. Several dyes and xenobiotics were reported as degraded by *Phanerochaete chrysosporium*; furthermore, these compounds were found to be mineralized to CO_2 to different degrees (Sparado et al., 1992). The correlation between LiP secreted by *Phanerochaete chrysosporium* and decolorization was indicated when decolorizations of azo, triphenyl methane, heterocyclic and polymeric dyes were observed. Most of the dyes lost over 75 percent of their color. The isolated LiP isozymes showed comparable ability to decolorize dyes

to that of crude *Phanerochaete chrysosporium* culture in the presence of veratryl alcohol, suggesting that LiP plays a major role in the decolorization, and that MnP is not necessarily required to start the degradation of these dyes (Ollika et al., 1993). C.I. Disperse Yellow 3 was also found efficiently decolorized by *Phanerochaete chrysosporium*; 4-methyl-1,2-benzoquinone and acetanilide were identified as degradation products. Surprisingly, a dimer of the initial dye molecule was also found (Sparado et al., 1994). Attempts have also been made to find out how aromatic substitution patterns can influence azo dye degradability by *Phanerochaete chrysosporium* (Pasti-Grigsby et al., 1992, Martins, M. et al., 2001). Nevertheless, the relationships between the substitution patterns and degradability are still not fully clear.

Thanks to the increasing recognition of *Phanerochaete chrysosporium*'s capability to decolorize various dyes, many other WRF have been studied for decolorization. Of the 18 strains of WRF studied by Heinfling et al., *Trametes versicolor and Bjerkandera adusta* demonstrated their effectiveness in decolorization of three azo dyes and two phthalocyanine dyes (Heinfling et al., 1997). Another WRF, *Irpex Lacteus,* was shown to completely decolorize six different groups of dyes (monoazo, diazo, anthraquinone, heterocyclic, triphenylmethane, phthalocyanine) within ten days (Novotny et al., 2001). Orange G was observed to be decolorized to a great extent by *Pleurotus sajorcaju* (Chagas and Durrant, 2001). *Thelephroa* sp. also decolorized Orange G, and a maximum of 61 percent decolorization of a dye industry effluent was also achieved by this fungus (Selvam et al, 2003).

1.3 Decolorization by *Pleurotus ostreatus* and its enzymes

Pleurotus ostreatus, a type of WRF, was early recognized for its ability to degrade Remazol Brilliant Blue R (Vyas and Molitoris, 1995). Out of 103 WRF strains, *Pleurotus*

ostreatus was found to be one of the top fungi to decolorize different dyes (Novotny et al., 2001). Cao also found that *Pleurotus ostreatus* as one of the most effective decolorizers of the nine WRF he studied (Cao, 2000).

Recent studies have increased the understanding of decolorization by *Pleurotus ostreatus*, the enzymes produced, and decolorization or degradation products. *Pleurotus ostreatus* has been regarded as a fungus belonging to an MnP/laccase group, which means that *Pleurotus ostreatus* does not produce LiP under any conditions (Hatakka, 1994). The only occasion in which LiP was detected in a *Pleurotu ostreatus* culture came from the study by Robinson and his colleagues (Robinson et al., 2001). In their enzyme assay experiments, veratryl alcohol was used as the substrate for LiP. Since both versatile peroxidase (VP) (Heinfling et al., 1998b) and veratryl alcohol oxidase (VAO) (Sannia et al., 1991) could possibly target veratryl alcohol as a substrate, the activity detected towards veratryl alcohol does not necessarily suggest that LiP is produced by *Pleurotus ostreatus*. In an investigation of decolorization of olive mill wastewater, substantial activities of both MnP and laccase were detected, whereas no LiP and VAO were detected (Aggelis et al., 2002). Early measurements of laccase activity were highly correlated with the final reduction of phenolic compounds in the wastewater. However, in another study on removal of phenolic pollutants by *Pleurotus ostreatus*, only laccase was detected. Hou's study also found that laccase was the only ligninolytic activity detected in the culture supernatant when decolorization of anthraquinone dye took place in the presence of *Pleurotus ostreatus* (Hou et al., 2004).

1.4 Parameters that effect the decolorization or degradation of textile dyes by WRF

Parameters such as pH, agitation and temperature can influence fungal physiology and the expression and activity of enzymes therefore affecting their efficiency to decolorize or degrade various organic pollutants, including dyes in textile waste water.

1.4.1 The effect of pH on decolorization or degradation of textile dyes by WRF

As one of the most important parameters for preparation of fungal decolorization culture, the effect of pH has been studied for many WRF. However, most of the attention has been paid to find the optimum pH to obtain highest activity towards certain substrates when using isolated enzymes. Research has shown such optimum pH's are both enzyme-dependent and substrate-dependent. Laccase from *Coriolus hirsutus* can achieve a highest activity toward ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] at about 2.5, while pH 4 was favored when DMP (2,6-dimethoxyphenol) was used as substrate (Shin, K. S. and Lee, Y. J., 2000). MnP from *Lentinula edodes* was shown to be quite stable in a broad pH range (4.0-6.0) with an optimum pH value of 4.5 toward substrate DMP (Boer, C. G. et al. , 2006). When MBTH (3-methyl-2-benzothiazolinone hydrazone) and DMAB (3,3-dimethylaminobenzoic acid) were used as substrates, the MnP produced by *Irpex lacteus* preferred an optimum pH of 5.5-6.5. (Baborova, P. et al., 2006). The effect of initial pH on the enzyme production and degradation of pollutants, including textile dyes, has been investigated by a few studies. In most of these studies, the final pH after decolorization or degradation differed from the initial values. In the case of decolorization of dye Poly R478 by five Zimbabwean WRF's, all fungal cultures showed small but consistent movement toward the optimum pH's (Tekere, M. et al., 2001). Radha's study also demonstrated that *Phanerochaete chrysosporium* cultures with different initial pH values all fell

in the range of 4.5 to 5 after maximum decolorizations were reached (Radha, K. V. et al., 2005). Production of some organic acids in many WRF secondary metabolisms can partially contribute to the pH self-adjustment by WRF (Wesenberg, D. et al., 2003). For instance, oxalic acid is often found to be produced by *Phanerochaete chrysosporium* as one of the small molecular weight mediators in its metabolism, which can lead to a pH decrease in the culture. However, productions of such organic acids are also restrained by the enzymes from WRF. LiP and MnP from *Phanerochaete chrysosporium* can help to decarboxylate oxalate upon catalysis, therefore preventing the excess accumulation of oxalate from interrupting the growth of hyphae (Makela, M. et. al., 2002, Popp, J. L. et. al., 1990, Akamatsu, Y., et al., 1990).

1.4.2 The effect of agitation on decolorization or degradation of textile dyes by WRF

The decolorization culture can be incubated under either static or agitated conditions. Wesenberg stated that, in general, production of two major enzymes from WRF, LiP and MnP, is generally optimal at high oxygen concentrations but is repressed by agitation in a submerged WRF liquid culture, while laccase production is often enhanced by agitation (Wesenberg et al, 2003). Static culture conditions have been reported to be necessary for the expression of the ligninolytic enzymes in *Trametes Versicolor*, *Phanerochaete chrysosporium* and *Pleurotus ostreatus* (Haapala, R and Linko, S. 1993, Johansson, T. and Nyman, P. O., 1987, Kim et al, 1996). Nevertheless, agitated cultures of *Phanerochaete chrysosporium* and *Trametes Versicolor* have decolorized pulp and olive mill effluents (Michel, F. C. et al., 1991, Sayadi, S. and Ellouz R., 1995). When incubated statically, WRF culture often form "mats" at the surface of growth medium. Such culture gave very little decolorization in both Jarosz et al's (2002) and Swamy's research (1999). When screening 115 fungi for their ability to decolorize Basic Blue 22

and Acid Red 183, Jarosz and his colleagues found that agitation was crucial to archieve a high level of decolorization by three selected fungi. In Swamy's study, cultures statically incubated only showed decolorization due to adsorption, while cultures initially incubated under agitation decolorized the dyes rapidly. Furthermore, in the same study, if a 10 day old static culture was agitated, the mycelium mat transformed into pellets and then an increase of decolorization was observed. Soares and Duran (1998) also reported that agitation was essential for achieving high rate of decolorization by WRF *Trametes villosa*. Glenn and Gold (1983) investigated the decolorization of several polymeric dyes by WRF, and they found that agitation was decisive too. The significantly higher decolorization efficiency of agitated cultures over static cultures was mainly due to the increased mass transfer between cells and the medium along with higher oxygen concentration in cultures (Swamy J. and Ramsay, J. A., 1999, Glenn and Gold, 1983).

However, the effect of agitation on decolorization depends not only on fungi species and enzyme production but also on dye structures. Ha et al (2001) reported the effect of agitation and oxygen upon the production of MnP and Laccase from *Pleurotus ostreatus*. Agitation greatly increased the production of MnP isozymes and triggered the formation of homologous pellets in culture, while not much difference in laccase production was observed between agitated and static cultures. The higher production of MnP in agitation cultures over static ones was considered primarily due to the higher oxygen concentration. A level of over 5 ppm of oxygen was necessary for MnP production. Nevertheless, in a study using *Dichomitus squalens*, *Ischnoderma resinosum* and *Pleurotus calyptratus* to decolorize Orange G and RBBR (Remazol Brilliant Blue R) (Eichlerova et al, 2005), shaken cultivation substantially inhibited laccase production for *Ischnoderma resinosum* and *Pleurotus calyptratus* for both dyes, while MnP production was not affected. Laccase activity also decreased in *Dichomitus squalens* grown in a

shaken culture in the presence of RBBR but increased in the presence of Orange G. The activity of MnP in *Dichomitus squalens* cultivated in the shaken culture decreased in the presence of both dyes. Definite conclusions have not yet been made about the enzymatic mechanism responsible for decolorization by WRF. One strain can use different mechanisms for the decolorization of different dyes (Eichlerova et al, 2005). Even though the ligninolytic degradation system of WRF has been believed to apply to the degradation of many xenobiotics (Wesenberg et al, 2003), the complexity of such degradations and involvement of many factors other than enzymes, such as free radicals, mediators, hydrogen peroxide, etc. have to be recognized and understood (Kotterman et al., 1996, Kapich et al., 1999 and Wesenberg et al, 2003).

1.4.3 The effect of temperature on decolorization or degradation of textile dyes by WRF

Different fungi have different optimal growth temperatures. Most of them grow at 25ºC, 30ºC or 35 ºC (Fu, Y., and Viraraghavan, T., 2001). In the decolorization of Astrazon Red FBL by *Funalia trogii*, Yesilada and colleagues (2002) found that 30ºC was the optimal temperature, while 35 ºC was proved to be the optimal temperature when several dyes in different structure classes were decolorized by *Phanerochaete chrysosporium* (Radha, K. V. et al., 2005). Ten types of WRF from semi-tropical forests were studied for their potential to decolorize dyes. Their optimum growth temperatures were determined to range from 25 ºC to 37 ºC, with each fungus having its individual optimum temperature (Tekere, M. et al., 2001).

1.5 Identification of decolorization or degradation products from textile dyes

As is known, decolorization or degradation of dyes may generate products more toxic than original dyes; therefore the degradation products from dyes should be investigated.

However, past studies have mostly focused on proving the ability of WRF to decolorize textile dyes and finding out the corresponding enzymes, rather than identifying the decolorization or degradation products.

Spadaro and Renganathan (1999) discovered a major product, acetanilide, from the degradation of Disperse Yellow 3. This dye was later studied by Zhao and Hardin (2006), and the same product was found in the degradation by *Pleurotus ostreatus*. In the case of indigo degradation by *Trametes Hirsuta* and *Sclerotium rolfsii*, anthranilic acid was identified as the major product (Campos R. et al., 2001). Among the few studies of the identification of degradation products of dyes by WRF, Zhao's work has established a relatively complete profile of routine procedures to analyze degradation products by GC/MS, HPLC and CE/MS (Zhao, X. et al, 2006, Zhao X. and Hardin, Ian R., 2006). The degradations of model compounds Disperse Orange 3 and Disperse Yellow 3 were studied. Nitrobenzene, 4-nitrophenol and 4-nitroaniline were identified as three major products from Disperse Orange 3, while acetanilide was found as a major product from the degradation of Disperse Yellow 3. In addition, 1-methoxy-4-nitrobenzene was found transformed from 4-nitrophenol through a methylation step (Zhao, X. et al, 2006, Zhao X. and Hardin, I .R., 2006). Such methlyation reactions were also observed in the degradation of 3,4-dichlorophenol by *Phanerochaete chysosporium* (Deschler, C. et al., 1998). Valli et al, (1992) reported similar methylation reactions in their study on degradation of 2,4-dinitrotoluene by *Phanerochaete chysosporium*; however, demethylation of the intermediate compounds, 1,2-dimethoxy-4-nitrobenzene was also discovered.

Even though decolorization or degradation of textile dyes by WRF has been extensively studied, knowledge on how culture parameters affect the fungi growth and therefore influence their efficiency or ability to decolorize and degrade textile dye is still fragmented. The specific

mechanism of such decolorization or degradation and the thorough pathway from dye to total mineralization are still not fully understood. Much more work has to be done in the future to find the answers.

1.6 Objectives of study.

1. Three water soluble dyes, Orange II, Acid Red 29 and Remazol Brilliant Blue, were used to study influence of buffer, pH, temperature and agitation on fungal growth and fungus' ability for decolorization. The optimal culture parameters were to be established and then used for the degradations of five nitrophenylazophenylamine azo dyes.
2. Five model nitrophenylazophenylamine dyes, Disperse Red 1, Disperse Red 19, Disperse Orange 1, Disperse Orange 25 and Disperse Red 13, were degraded by *Pleurotus ostreatus*. GC/MS (Gas chromatography/mass spectrometry) and HPLC (High performance liquid chromatography) were used to identify the degradation products from the five dyes. Research was also done to study how the initial dye concentration might affect the degradation results. The degradation pathway of the dyes was explained.
3. Further breakdown of the major degradation products from the dyes was studied in detail. The possible effect of initial concentration of compounds was also studied. The pathway of the degradation of the major products was explained.

CHAPTER 2

MATERIAL AND METHODS

2.1 Materials

2.1.1 Chemicals

Three water soluble azo dyes were used in this study. Orange II (4-(2-hydroxy-1-naphthylazo)benzenesulfonic acid sodium salt, Color Index No. 15510, colorant content >85%) and Acid Red 29 (2-(phenylazo)chromotropic acid disodium salt, Color Index No. 16570, 75% colorant content) were purchased from Aldrich Chemical Co. (Milwaukee, Wis.). Remazol Brilliant Blue R (1-amino-4-[m-(2-hydroxyethylsulfonyl)anilino]-hydrogen sulfate disodium salt, Color Index No. 61200, 50% colorant content) was purchased from Sigma (St. Louis, MO). Five nitrophenylazophenylamine dyes were chosen this study. These dyes were Disperse Red 1 (*N*-ethyl-*N*-(2-hydroxyethyl)-4-(4-nitrophenylazo)aniline, Color Index No. 11110, 95% colorant content), Disperse Red 19 (4'-[(N,N-dihydroxyethyl)amino]-4-nitroazobenzene, Color Index No.11130, 95% colorant content), Disperse Orange 25 (3-[*N*-ethyl-4-(4-nitrophenylazo)phenylamino]propionitrile, Color Index No. 11227, 95% colorant content) and Disperse Red 13 (2-[4-(2-chloro-4-nitrophenylazo)-*N*-ethylphenylamino]ethanol, Color Index No. 11115, 95% colorant content) were purchased from Aldrich Chemical Co. (Milwaukee, Wis.). Disperse Orange 1 (4-(4-nitrophenylazo)diphenylamine, Color Index No. 11080, 99% colorant content) was purchased from M.P. Biochemical LLC. (Irvine, CA).

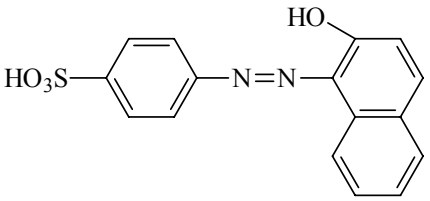
Standard compounds used were nitrobenzene, 4-nitrophenol, 4-nitroaniline, 4-nitroanisole, 4-nitrocatechol, 4-nitroguaiacol, 1,2-dimethoxy-4-nitrobenzene, 2,4-dinitrophenol,

2,4-dinitroanisole, 3-chloronitrobenzene, 2-chloro-4-nitroaniline, 2-choro-4-nitrophenol, 2-chloro-4-nitroanisole, veratryl alcohol and veratryl aldehyde. All of these compounds were analytical grade and purchased from Sigma or Aldrich, except 2-chloro-4-nitroanisole, which was purchased from Matrix Scientific (Columbia, SC).

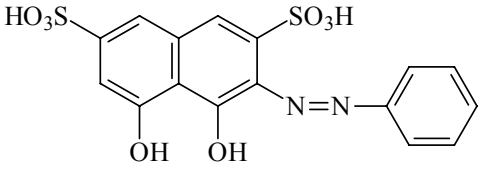
Solvents used for HPLC were acetonitrile and water of HPLC grade (J. T. Baker, Phillipsburg, NJ). Phosphoric acid and sodium hydroxide pellets were analytical grade (J. T. Baker, Phillipsburg, NJ). All other chemicals used throughout this study were reagent-grade chemicals. Purified water was from a filtration system provided by U.S. Filter (Warrendale, PA).

Table 2.1 Dyes used in this study

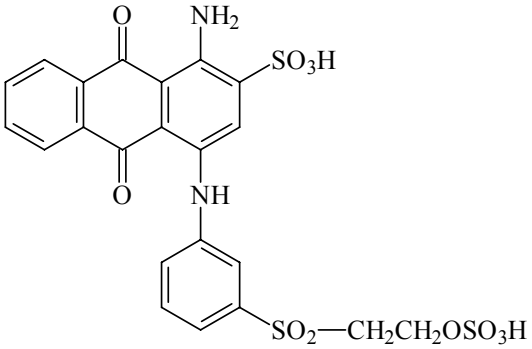
Dye Names	Chemical Names	Color Index No.	CAS No.
Orange II	4-(2-hydroxy-1-naphthylazo) benzenesulfonic acid sodium salt	15510	633-96-5
Acid Red 29	2-(phenylazo)chromotropic acid disodium salt	16570	4197-07-3
Remazol Brilliant Blue R (RBBR)	(1-amino-4-[m-(2-hydroxyethylsulfonyl)anilino]-hydrogen sulfate disodium salt	61200	2580-78-1
Disperse Red 1	*N*-ethyl-*N*-(2-hydroxyethyl)-4-(4-nitrophenylazo)aniline	11110	2872-52-8
Disperse Red 19	4'-[(N,N-dihydroxyethyl)amino]-4-nitroazobenzene	11130	2734-52-3
Disperse Orange 1	4-(4-nitrophenylazo)diphenylamine	11080	2581-69-3
Disperse Orange 25	3-[*N*-ethyl-4-(4-nitrophenylazo) phenylamino]propionitrile	11227	31482-56-1
Disperse Red 13	2-[4-(2-chloro-4-nitrophenylazo)-*N*-ethylphenylamino]ethanol	11115	3180-81-2



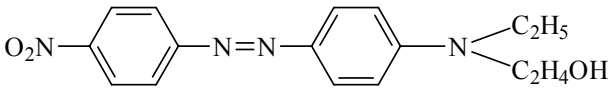
Orange II



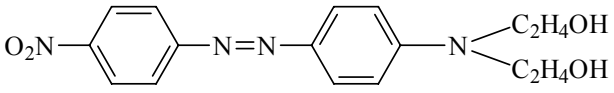
Acid Red 29



Remazol Brilliant Blue R (Reactive Blue 19)

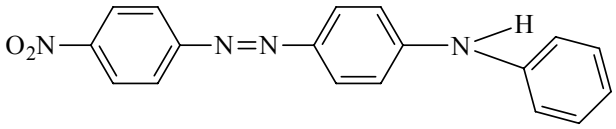


Disperse Red 1

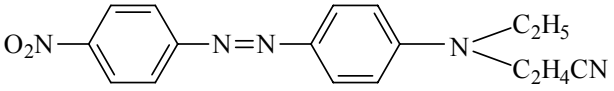


Disperse Red 19

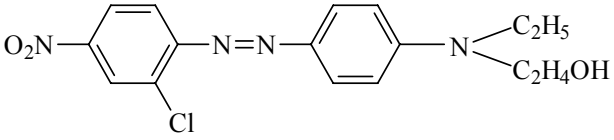
Figure 2.1 Structure of the dyes used in this study



Disperse Orange 1



Disperse Orange 25



Disperse Red 13

Figure 2.1 continued

2.1.2 Filters

Three types of filters were tested for the possible adsorption of the water soluble dyes and degradation products from hydrophobic dyes. They were nylon (polyamide), PVDF (polyvinylidene fluoride) and PTFE (polytetrafluoroethylene) filters. For three water soluble dyes, the color of dye solutions (0.1g/L) was measured by a spectrophotometer before and after filtration with the three types of filters. Standard solutions (0.1g/L) of four possible degradation products, nitrobenzene, 4-nitrophenol, 4-nitroaniline and 4-nitroanisole were prepared with methanol/ water (50/50). The solutions were filtered with three types of filters. The concentrations of the solutions, before and after filtration, were determined on a HPLC system, which is described in detail in the "methods" section. The nylon filter showed apparent adsorption of dyes, while the PVDF and PTFE filters exhibited very little dye adsorption.

Likewise, nylon filters retained a great amount of the four possible degradation products, while both PVDF and PTFE showed very little adsorption. Therefore, the nylon filter was eliminated. PVDF was finally chosen over PTFE because of the lower price and higher hydrophilicity.

Table 2.2 Recovery Tests of the water soluble dyes and possible degradation products on three types of filters

Dyes and possible degradation products.	Compounds recovery percentage (%)		
	NYLON	PVDF	PTFE
Orange II	86.7	98.2	96.5
Acid Red 29	97.9	99.9	99.9
RBBR	93.0	99.3	100
Nitrobenzene	92.1	97.9	99.0
4-Nitrophenol	80.1	100	100
4-Nitroaniline	84.0	100	100
4-Nitroanisole	88.4	98.6	99.1

2.1.3 Microorganism

Pleurotus ostreatus (Florida strain), was used in this study. This fungus was obtained from the Dr. Karl-Erik Eriksson's laboratory at the University of Georgia. The culture was maintained on malt agar plates (malt extract 20 g/L, agar 15 g/L) at 30°C in a temperature chamber. The fungus was subcultured every five days when the mycelium growth reached the edge of the agar plate.

2.1.4 Preparation of the decolorization and degradation culture

An entire 5 days old agar plate of fungus was added into 200ml Kirk's Medium (Table 2.3) and blended to reach homogeneity by a bio-homogenizer (Biospec Products Inc., Bartlesville, OK). Five ml of the homogenous mixture was added to a 125 ml autoclaved Kirk's medium aliquot. The pH value was adjusted to 5.0 with acetic acid and sodium hydroxide in an

Erlenmeyer flask. In this study of the effect of different initial pH on decolorization of three water soluble dyes, pH 3, 4 and 5 solutions were prepared with acetic acid and sodium hydroxide, while pH 6 and 7 solutions were prepared with potassium dihydrogenphosphate and sodium hydroxide. Cultures were incubated at 30ºC and 200 rpm for 3 days before the addition of dyes or other compounds.

Table 2.3 Composition of Kirk's Medium (Kirk et al., 1978)

CHEMICALS	CONCENTRATION (G/L)
Potassium dihydrogenphosphate	0.2
Magnesium sulfate	0.05
Calcium chloride	0.01
Ammonium tartrate	0.22
Glucose	10
Nitrilotriacetate	1.5e-3
$MgSO_4 \cdot 7H_2O$	3.0e-3
$MgSO_4 \cdot H_2O$	5.0e-4
NaCl	1.0e-3
$FeSO_4 \cdot 7H_2O$	1.0e-4
$CoSO_4$	1.0e-4
$ZnSO_4$	1.0e-4
$CuSO_4 \cdot 5H_2O$	1.0e-5
$AlK(SO_4)_2$	1.0e-5
H_3BO_3	1.0e-5
$NaMoO_4$	1.0e-5
Biotin	1.0e-3
Folic acid	2.5e-3
Thiamine• HCl	2.5e-3
Riboflavin	2.5e-3
Pyridoxine• HCl	5.0e-3
Cyanocobalamine	5.0e-5
Nicotinic acid	2.5e-3
DL-calcium pantothenate	2.5e-3
p-Aminobenzoic acid	2.5e-3
Thioctic acid	2.5e-3

2.2 Methods

2.2.1 Decolorization of three water soluble dyes

Stock dye solutions were prepared at 4g/l. After 3 days incubation of cultures, 3.3 ml of dye stock solution was added to the culture to give an initial concentration of 100 ppm. The temperature and shaking speed were fixed to 30ºC and 150 rpm after the addition of dyes. Controls were carried out without dyes or fungus.

2.2.2 Degradation of the five hydrophobic azo dyes and their degradation products

Well-grounded powders of the dyes or product compounds were weighed on a microbalance and added to the grown culture. The temperature and shaking speed were fixed to 30ºC and 150 rpm after the addition of dyes. Controls were carried out without dyes, product compounds or fungus.

2.2.3 The pH measurements of fungal cultures

The pH values of fungal cultures during incubation and decolorization were measured every 24 hours with a pH meter (Beckman Coulter, Inc., CA).

2.2.4 Color measurement for decolorization of three water soluble dyes

Two ml of decolorization culture was sampled and filtered with a 0.45μm PVDF filter. One ml of filtrate was mixed with 1 ml of deionized water and the color was measured on a spectrophotometer (Shimazu, UV-2401PC). The area under the absorption curve in the visible range, from 300nm to 700nm, was recorded. All reported results were based on 2 or 3 replicates.

2.2.5 Bioadsorption of three water soluble dyes on the fungal mass

After 3 days incubation, the fungal cultures were autoclaved. Stock solutions of three ionic dyes were added into the autoclaved cultures to give an initial dye concentration of 100ppm. The cultures were shaken at 150ppm for 10 minutes, and the color of these cultures was measured on a spectrophotometer for the starting color. Then, the color of cultures was measured 24, 48 and 72 hours after the dye addition. Color reduction in the cultures was considered to represent the bioadsoprtion of the dyes on the fungal mass. The results were based on three replicates.

2.2.6 High performance liquid chromatography (HPLC) analysis of the degradation of five hydrophobic azo dyes

One ml of degradation culture was taken and filtered with a 0.45µm PVDF filter. The filtrate was maintained in sealed sample vial. Analysis by a Hewlett-Packard 1100 series HPLC system (Hewlett-Packard GmbH, Germany), consisting of a model G1311A quaternary pump, G1322A degasser, and a diode array detector (Model G1315A). HP ChemStation software (version B.01.03) was used for data processing and reporting. The injection volume was set to at 100µl with an automatic injector (Model 1313A). The flow rate was held at 1 ml/min during the run. The HPLC program is presented in Table 2.4. Each analyte was monitored at its maximum absorption wavelength and the peak area was used to represent the amount of the compound.

Table 2.4 HPLC program for analysis of the degradation products

Time (min)	Flow Rate (ml/min)	%A	%B
0	1	5.0	95.0
20	1	25.0	75.0
30	1	60.0	40.0
40	1	60.0	40.0
45	1	5.0	95.0
50	1	5.0	95.0

A: Acetonitrile
B: 0.025M Phosphate Buffer, pH=3.0.

The identification of products was done by the comparison of retention time and UV spectrum with standard compounds. The results were based on three replicates.

2.2.7 Gas chromatography/mass spectrometry (GC/MS) anaylsis of the degradation of five hydrophobic azo dyes

Ten ml of degradation culture was sampled and extracted with 20 ml methylene chloride three times. The combined organic layers, approximately 60 ml, were concentrated to about 0.5 ml by a rotation evaporator (BÜCHI, Rotavapor R-124).

GC/MS was performed with a GC-17A gas chromatograph coupled with a QP5000 mass spectrometer from Shimadzu (Kyoto, Japan). The ionization voltage was 70 eV. Gas chromatography was conducted in the temperature-programming mode with a XTI-5 column (0.25 mm by 30 m) from Restek (Bellefonte, PA). The initial column temperature was held at 40 °C for 4 min, then increased linearly to 270°C at 10 °C/min, and held for 5 min at 270°C. The temperature of the injection port was 275 °C and the GC/MS interface was maintained at 290°C. Helium was used as carrier gas with a flow rate of 1.0 ml/min. The injection mode was set to be splitless. Identification of degradation products was made by comparison of retention time and fragmentation pattern with standard compounds as well as with mass spectra in the NIST spectral library stored in the computer software (version 1.10 beta, Shimadzu) of the GC/MS. The results were based on 2 or more replicates.

2.2.8 Biomass measurement

Decolorization culture was filtered through pre-weighed wool glass under reduced pressure. Fungal pellets were retained on glass wool and dried in an oven at 90°C for 48 hours. The dried mass was measured on a balance. The results were based on three replicates.

2.2.9 Protein concentration

Bradford reagent was used to determine the protein concentration in cultures. The Reaction mixture was composed of 1ml Bradford reagent and 1ml sample filtrate or standard protein solution. The mixture was vigorously shaken and kept still for 15 min before the measurement. The protein concentration was determined by the absorption of reaction mixture at 595nm (Sigma, Product specification, 2006). The calibration curve was plotted by measuring BSA (Bovine serum albumin) standard solutions at 0, 1, 2, 4, 6, 8 and 10 μg/l. The results were based on three replicates.

2.2.10 Enzyme activity assay

Three enzyme assays were performed targeting the possible existing enzymes in the culture. They were LiP (lignin peroxidase), MnP (manganese peroxidase) and laccase. LiP assay mixture contained 1ml of 100 mM sodium tartrate buffer (pH 3.0), 0.5 ml of 10mM veratryl alcohol, 0.5 ml of 2mM hydrogen peroxide and 1ml of culture filtrate. Reaction started with the addition of hydrogen peroxide. The increase of absorption was monitored at 310nm (extinction coefficient = 9300 cm^{-1} M^{-1}). The assay of versatile peroxidase (VA) was done using the same reaction mixture but without hydrogen peroxide.

The MnP assay mixture contained 0.5 ml of 10 mM manganese sulfate, 0.5 ml of 2 mM hydrogen peroxide, 1 ml of 100 mM sodium malonate buffer (pH 4.5) and 1 ml of culture filtrate. The reaction was started with the addition of hydrogen peroxide. The increase of absorption was monitored at 270 nm (extinction coefficient = 11590 cm^{-1} M^{-1}).

The laccase assay mixture contained 0.5 ml of ABTS solution, 1.5 ml of 100 mM sodium acetate buffer, 1ml of culture filtrate. The reaction was started with the addition of ABTS. The increase of absorption was monitored at 420 nm (extinction coefficient = 36000 cm^{-1} M^{-1}). In all three assays, the initial linear portion of the time-absorption curve was used to calculate the enzyme activity. One unit of activity is defined as the amount of enzyme that can convert 1μ mol substrate per minute. All results were based on three replicates.

2.2.11 ANOVA analysis

ANOVA single factor analysis was performed on the 3rd day decolorization data for the three water soluble dyes to determine if buffer, initial pH, agitation and temperature had a significant influence on decolorization rate. When the P-value is larger than 0.05, it is concluded that the corresponding parameter does not have a significant effect on decolorization. If a P-value is less than 0.05, then it is concluded that the culture parameter has a significant effect on decolorization.

CHAPTER 3

THE EFFECT OF CULTURE PARAMETERS ON DECOLORIZATION OF THREE WATER SOLUBLE DYES

Research has shown that buffer, pH, agitation and temperature play significant roles in fungal growth and subsequent decolorization or degradation. In this study, the five nitrophenylazophenylamine dyes are all sparingly soluble in water, which makes it quite complicated to measure the actual amount of dye that is degraded. Therefore, three water soluble dyes, Orange II, Acid Red 29 and Remazol Brilliant Blue R were used to study the effect of buffer, pH, agitation and temperature on fungal growth and fungus' ability for decolorization. Previous study in our group has shown that *Pleurotus ostreatus* can effectively decolorize these three dyes, and the effect of the culture preparation parameters can be easily shown by monitoring the decolorization rates. The optimal degradation conditions was established according to the results of the decolorization of the three water soluble dyes and then used for the degradation of five nitrophenylazophenylamine azo dyes, which is discussed in Chapter 4.

3.1 Buffer concentration in culture and its influence on decolorization.

Various fungi have been found to be able to produce mediators, often organic acids, during their metabolic cycles. Due to, but not limited to, the existence of these organic acids, the pH of the fungal cultures change during decolorization or degradation. Therefore, the initial buffer concentration of the fungal culture can greatly affect the decolorization or degradation by influencing the extent that the culture needs adjustment of the pH.

In the preparation of the *Pleurotus ostreatus* culture, different amounts of acetic acid were added. Sodium hydroxide solution was used to titrate the cultures to pH 5. Cultures with different buffer concentrations were prepared. The buffer concentrations studied were 0, 10 mM, 20 mM and 30mM. All three water soluble dyes were studied for the influence of buffer concentration on their decolorizations. Color measurement was taken every 24 hours till the seventh day. The initial dye concentration of Orange II was 100 ppm. The pH values were monitored from the first day of incubation, and are presented in Figure 3.1. Color measurement began after the addition of dye, which was after 3 days culture incubation (Figure 3.2).

The cultures without buffer did not give any decolorization, and pH's dropped rapidly to about 3.0 after 3 days incubation. A pH between 4 and 5 has been considered optimal for enzymes from *Pleurotus ostreatus* to function. Thus, given a pH as low as 3, the culture with no buffer was not able to either produce any enzymes or provide a suitable pH level for enzymes to work. Cultures having 10 mM buffer removed about 90 percent of the color within 3 days. Surprisingly, an increase followed by a significant decrease in pH value was seen in cultures having 10 mM buffer during the incubation. However, an explanation of such phenomenon could not be given. Cultures with 20 mM buffer exhibited the same efficiency in decolorization as those with 10 mM buffer, but without same variation in pH. Cultures having 30 mM buffer eventually achieved about 90 percent color removal after 4 days, which was one day more than cultures with 10 mM and 20 mM buffers. This is possibly due to the increased difficulty for the fungus to adjust its culture pH under the higher buffer concentration. The low degree of

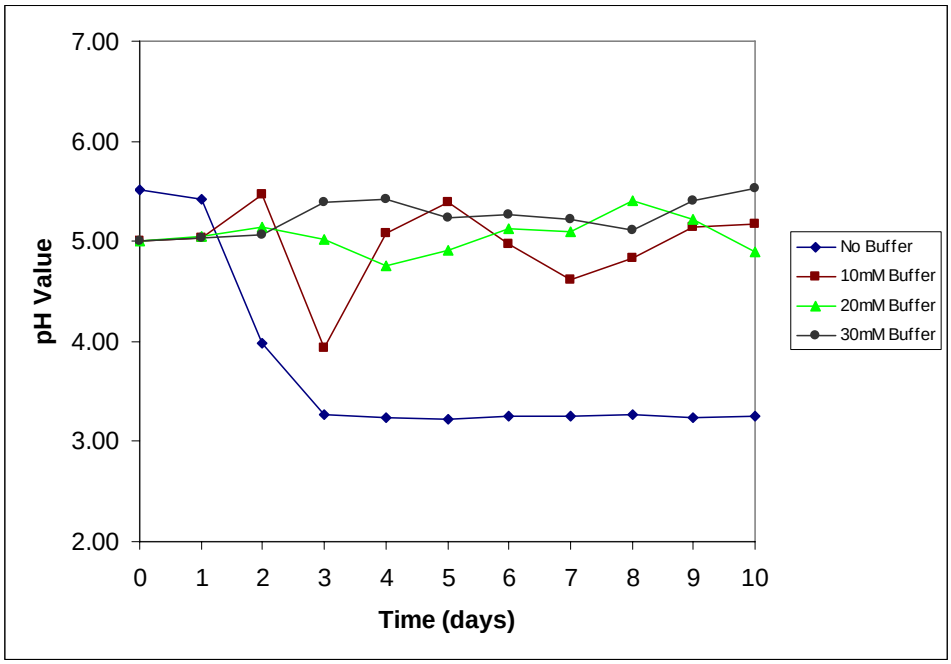


Figure 3.1 Change in pH for Orange II decolorization with different buffer concentrations
Dye was added to the culture on 3rd day

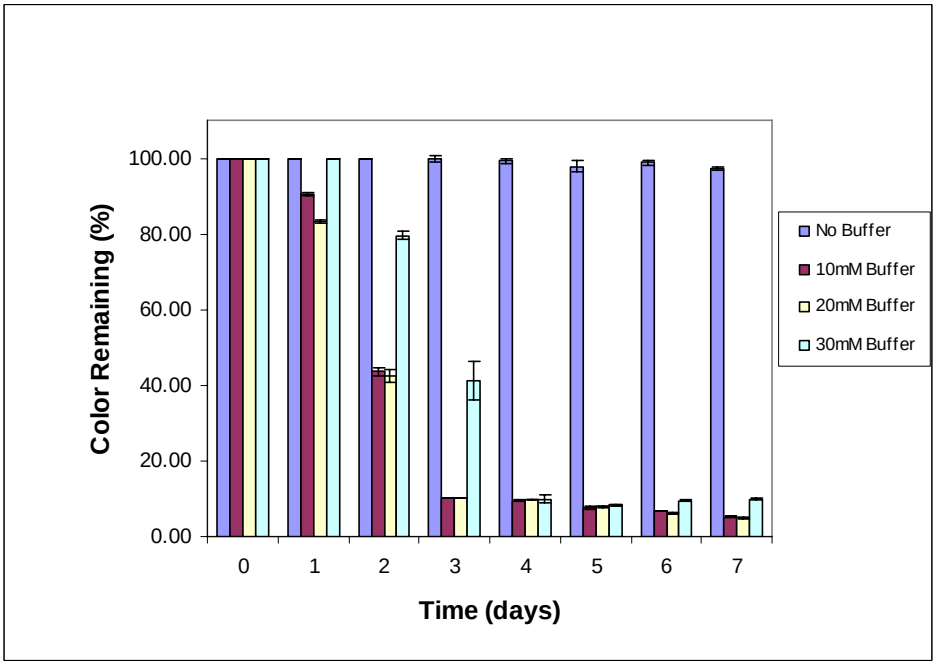


Figure 3.2 Effect of buffer concentration on Orange II decolorization

fluctuation of pH in 30 mM buffer cultures seems to confirm this. These results indicated that buffer concentration played a significant role in decolorization of Orange II, which was supported by the ANOVA analysis results (refer to Appendices). When data for 3 day Orange II decolorization cultures with different buffer concentration were tested by ANOVA, a P-value of 4.44×10^{-5} was obtained, which indicated that the means of the 3 day decolorization rate were significantly different due to different buffer concentration.

The bio-parameters, including protein concentration and enzyme activities were measured. The biomass of 10 mM and 20 mM buffer cultures, which showed a faster rate of decolorization than the other two, were higher than the other two conditions (Table 3.1). However, the 30 mM buffer samples, which had the least biomass, still reached a high level of decolorization. The protein concentrations of 10 mM and 20 mM buffer samples were about half of that of 30 mM cultures after 7 days decolorization, but their ability to decolorize Orange II was apparently stronger, especially within the first 3 days of decolorization

Table 3.1 Bio-parameters of 7 days old Orange II decolorization cultures at different buffer concentrations.

Bio-parameters	No Buffer	10 mM buffer	20 mM buffer	30 mM buffer
Biomass (g)	0.115 ± 0.003	0.176 ± 0.006	0.171 ± 0.023	0.007 ±0.001
Protein concentration	1.13 ± 0.13	5.52 ± 0.34	4.14 ± 1.28	11.7 ± 0.1
LiP activity (U/L)	0	12.5 ± 0.9	4.25 ± 3.85	3.05 ± 1.55
MnP activity (U/L)	0	47.1 ± 6.3	12.5 ± 9.6	79.1 ± 12.6
Laccase activity	0	50.4 ± 3.3	12.5 ± 10	45.3 ± 8.8

Both MnP and laccase activities were detected in all cultures that had buffers, regardless of the concentration. However, no clear correlation was found between the decolorization rate

and level of enzyme activities. Ten mM buffer samples produced much more MnP and laccase than 20mM buffer samples. Nevertheless, there was no obvious difference between these two in decolorization rate, and the highest level of MnP and second highest laccase activity did not cause the 30 mM buffer to give the faster decolorization. Since these bio-parameters were measured after 7 days of decolorization, they may not reflect the actual changes of bio-parameters during the whole period. But due to the limited capacity of our shakers and amount of culture aliquot for sampling, we did not sample the decolorization cultures for daily measurement of bio-parameters.

According to a previous report, *Pleurotus ostreatus* does not produce LiP (Wesenberg et al, 2003). Using the veratryl alcohol and hydrogen peroxide assay, we detected the "LiP" activity. However, this did not necessarily indicate that LiP was produced by *Pleurotus ostreatus*. Since both the VP (Heinfling, et al., 1998b) and veratryl alcohol oxidase (VAO) (Sannia et al., 1991) could likely take veratryl alcohol as their substrate even without hydrogen peroxide, the activity detected towards veratryl alcohol does not definitely mean that LiP was produced by *Pleurotus ostreatus*. In the 7 day decolorization cultures (10mM buffer) of Orange

Table 3.2 Test of possible activity of laccase (from *Tramete versicolor)* to LiP and MnP assays

Sample	Laccase (U/L)	LiP (U/L)	MnP (U/L)
Pure laccase solution	59.4	28.2	0
Orange II 7 day decolorization culture with 10mM buffer	50.4	12.5	47.1

II samples, we did detect about 16 U/L activity towards veratryl alcohol without the presence of hydrogen peroxide. Later, a commercially available laccase from *Trametes versicolor* was used

to test the possible activity of laccase towards LiP and MnP assays. The results (Table 3.2) showed that laccase from *Trametes versicolor* clearly showed activity in the veratryl alcohol-hydrogen peroxide assay, but no activity in the MnP assay as we used. Therefore, the detected LiP activity was possibly due to the existence of VP or VAO and the interference from laccase in the *Pleurotus ostreatus* cultures.

When pH values and color removal were monitored during decolorization of Acid Red 29 in cultures with different buffer concentrations, results similar to those of Orange II were obtained (Figure 3.3 and 3.4). A P-value of 1.41×10^{-6} was calculated when the 3 day decolorization rate of Acid Red 29 was tested (refer to Appendices). This indicated, as in the case of Orange II, that buffer concentration can significantly influence the decolorization of Acid Red 29.

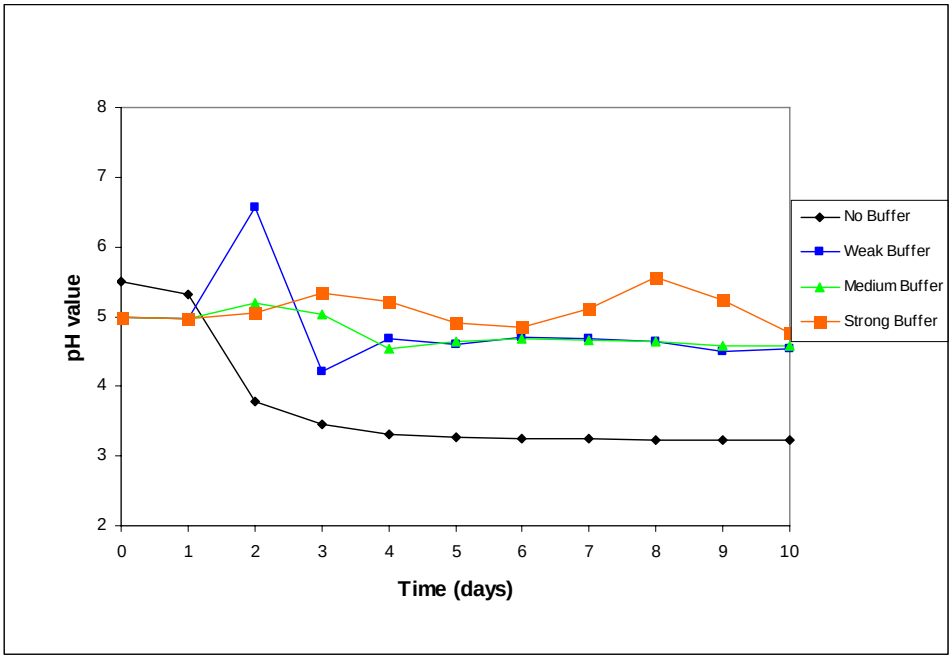


Figure 3.3 Effect of buffer concentration on pH values of the Acid Red 29 decolorization
Dye was added to the culture on 3rd day

A considerable pH decrease was shown for cultures without buffer, and such cultures again did not successfully decolorize the dye. Cultures having 10 mM and 20 mM buffer all showed great degree of decolorization, while 10 mM ones presented a slightly faster decolorization rate over 20 mM ones. Unlike the case of Orange II, cultures with 30 mM buffer could only remove about 30 percent of the total color even after 7 days decolorization.

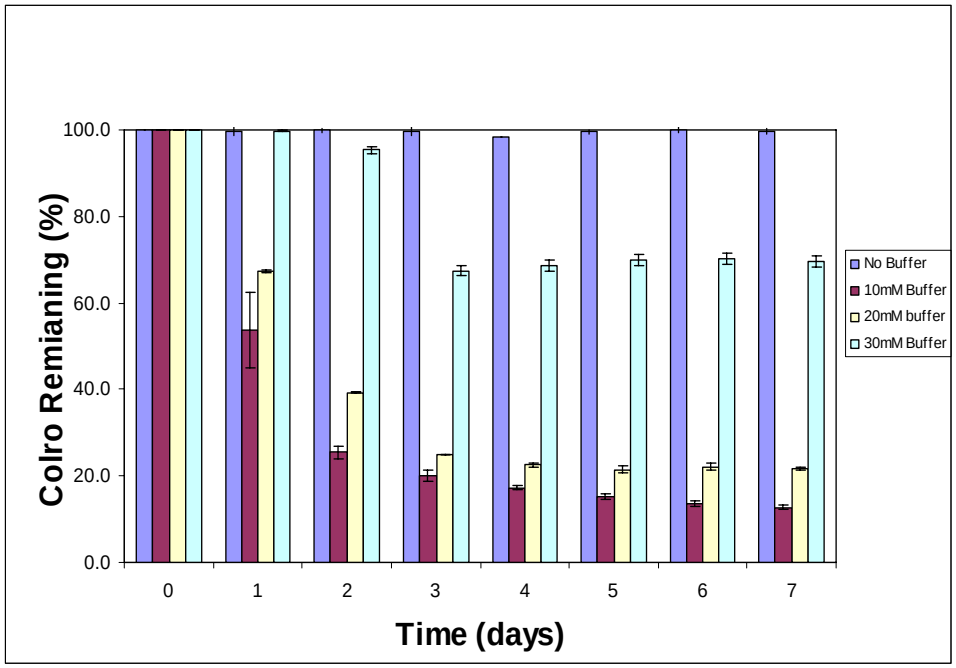


Figure 3.4 Effect of buffer concentration on Acid Red 29 decolorization

Bio-parameters were also measured for 7 day Acid Red 29 decolorization cultures with different buffer concentrations (Table 3.3). Ten mM and 20 mM buffer samples gave the highest biomass in accordance with the fastest rates of decolorization. However, 30 mM buffer samples had the highest protein concentrations but did not achieve satisfactory decolorization. The results of the enzyme assays did not provide a clear explanation of the relationship between enzyme production and decolorization. Even though the fastest decolorizing cultures were the

ones that had the most enzymes, the second fastest decolorized cultures with 20 mM buffer exhibited only slight MnP acitivity. As discussed earlier, the LiP acitivity detected did not necessarily indicate the existence of LiP in the *Pleurotus ostreatus* culture.

Table 3.3 Bio-parameters of 7 day Acid Red 29 decolorization cultures

Bio-parameters	No Buffer	10 mM buffer	20 mM buffer	30 mM buffer
Biomass (g)	0.156 ± 0.003	0.323 ± 0.035	0.271 ± 0.021	0 .120 ± 0.002
Protein concentration	0.346 ± 0.126	1.827 ± 0.120	1.528 ± 0.089	2.011 ± 0.034
LiP activity (U/L)	0	3.195 ± 0.745	0	0
MnP activity (U/L)	0	3.395 ± 0.285	0.775 ± 0.775	0
Laccase activity	0	3.825 ± 0.325	0	0

Comparing the results for Orange II and Acid Red 29, it was found that 10 mM and 20 mM buffer concentrations gave the best decolorizations. Cultures without buffer did not decolorize either dye at all. A 30 mM buffer culture decolorized more than 90 percent of Orange II but less than 40 percent of Acid Red 29. A definite relationship could not be found between enzyme production and decolorization efficiency. The enzyme production for Acid Red 29 cultures was much lower than in Orange II decolorization cultures. However, substantial decolorization of Acid Red 29 still occurred in the 10 mM and 20 mM buffer cultures, even though the 20 mM buffer culture produced very little MnP while decolorizing Acid Red 29. These decolorization and bio-parameters results again illustrated the complexity of the ligninolytic fungal degradation system. The ligninolytic enzymes were not the only factor involved. Many other factors, such as mediators, hydrogen peroxide, oxygen concentration all

play important roles in such decolorization systems. Experiments were also carried out to isolate the enzymes from the crude decolorization cultures by using FPLC and SDS-PAGE. However, the experiments did not produce satisfactory results. Due to the lack of relationship between bio-parameters and decolorization efficiency, bio-parameters were not measured for the follow-up experiments. Five days were sufficient to show clear differences in the decolorizations under different conditions, and the observation period was shortened from 7 days to 5 days.

Remazol Brilliant Blue R (RBBR) is one of the textile dyes that have extensively studied for fungal decolorization, especially for *Pleurotus ostreatus*. However, the effect of culture preparation parameters on RBBR decolorization has not been studied extensively. Figures 3.5 and 3.6 show the pH values during decolorization of RBBR under different buffer concentrations. The pH values changes were parallel to those in Acid Red 29 decolorization cultures. Cultures with no buffer gave almost no decolorization, while 30 mM buffer showed very poor decolorization capability. Twenty mM buffer exhibited the fastest decolorization rate among the four conditions. In spite of a lower decolorization rate during the first three days, cultures having 10 mM buffer reached same degree of color removal as 20 mM ones after 4 days. A P-value of 4.15×10^{-5} was obtained (refer to Appendices), which indicated buffer concentration played a significant role in the decolorization of RBBR.

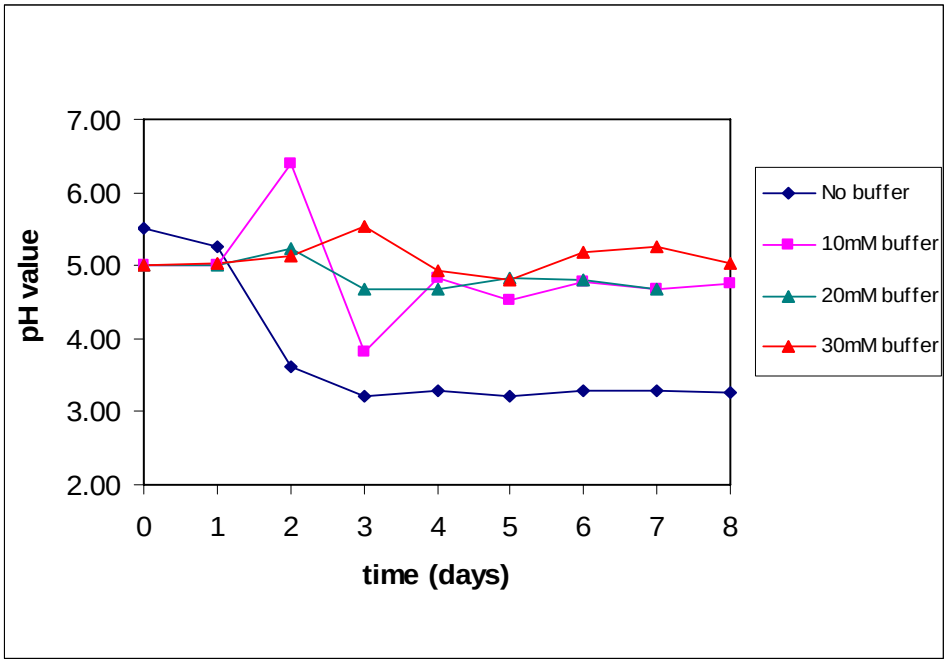


Figure 3.5 Change in pH values for the RBBR decolorization with different buffer concentrations Dye was added to the culture on 3rd day

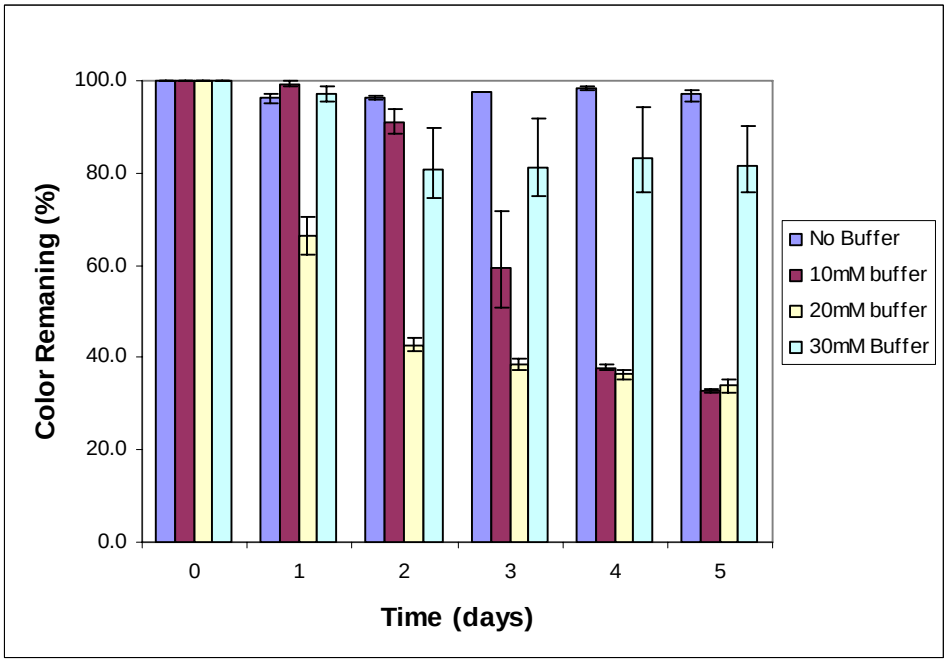


Figure 3.6 Effect of buffer concentration on the RBBR decolorization

Given these results, it is concluded that buffer concentration in fungal cultures preparation played a very significant role in decolorization ability and efficiency. Without any buffer, the pH of fungal cultures tended to quickly decrease to about 3, under which condition either no enzymes were produced or the enzymes were not able to work properly. High buffer concentration was also not preferred as for decolorization. This was likely due to the increased difficulty for the fungus to adjust its own growth environment. Both 10 mM and 20 mM buffers were suitable for decolorization. After 4 days, they both reached about same level of decolorization. The pH values of pure cultures without the addition of dyes at different buffer concentration were also recorded. The results showed no effect on pH was from the addition of dyes (Figure 3.7)

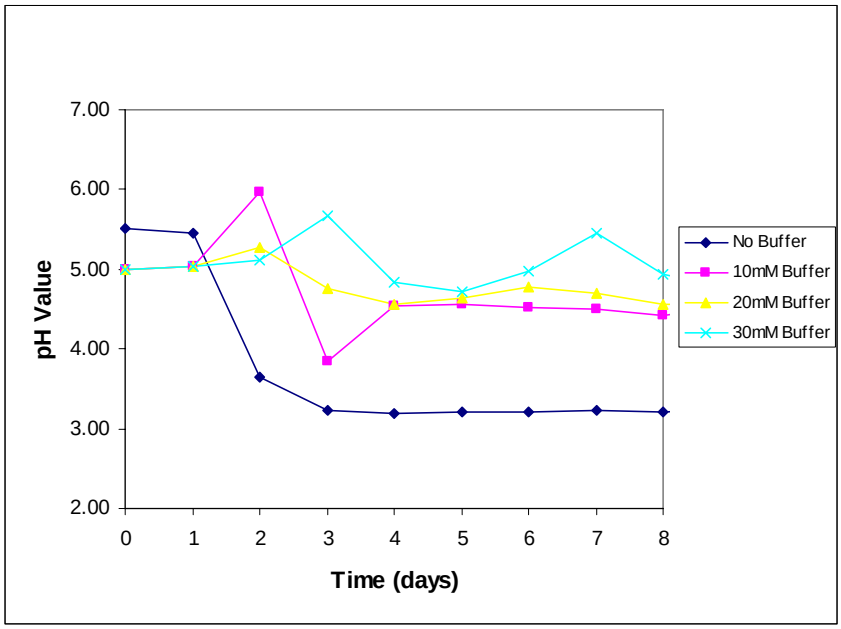


Figure 3.7 Effect of buffer concentration on pH values of pure cultures

3.2 Effect of initial pH of culture on decolorization of three water soluble dyes.

Major enzymes produced by white rot fungi all have optimum pH levels for highest activities. The pH values in culture preparation indeed exhibit a significant influence on decolorization and degradation by white rot fungi, even though fungi, by themselves, can adjust the growth environment to certain degrees. Therefore, the effect of the initial pH of cultures on the decolorization of three water soluble dyes was studied. Buffers were prepared at 10 mM for Orange II and Acid Red 29, while 20 mM buffer were used for RBBR according to the results discussed in section 3.1.

As with Orange II, only pH 5 and 7 demonstrated satisfactory decolorization, while cultures with the initial pH at 3, 4 and 6 did not show more than 10 percent color removal (Figure 3.9). ANOVA analysis gave a P-value of 2.63×10^{-10} for 3 day decolorization results, which indicated that the initial pH can significantly affect the decolorization rate (refer to Appendices). In the case of pH 5 and 7, after three days incubation the cultures had reached pH values around 5 (Figure 3.8), which was in the optimal range for the enzymes (MnP or/and laccase) from *Pleurotus ostreatus* to show high activities. Cultures having an initial pH of 6 showed a quick decrease in pH within 3 days incubation, and the pH did not rise above 4 afterwards (Figure 3.8). No apparent pH changes were observed for cultures having initial pH's of 3 and 4. These results indicated that a pH of 5 was essential for *Pleurouts ostreatus* to decolorize Orange II. A high acidity, either initially imposed or being reached after incubation, will prohibit a large degree of decolorization.

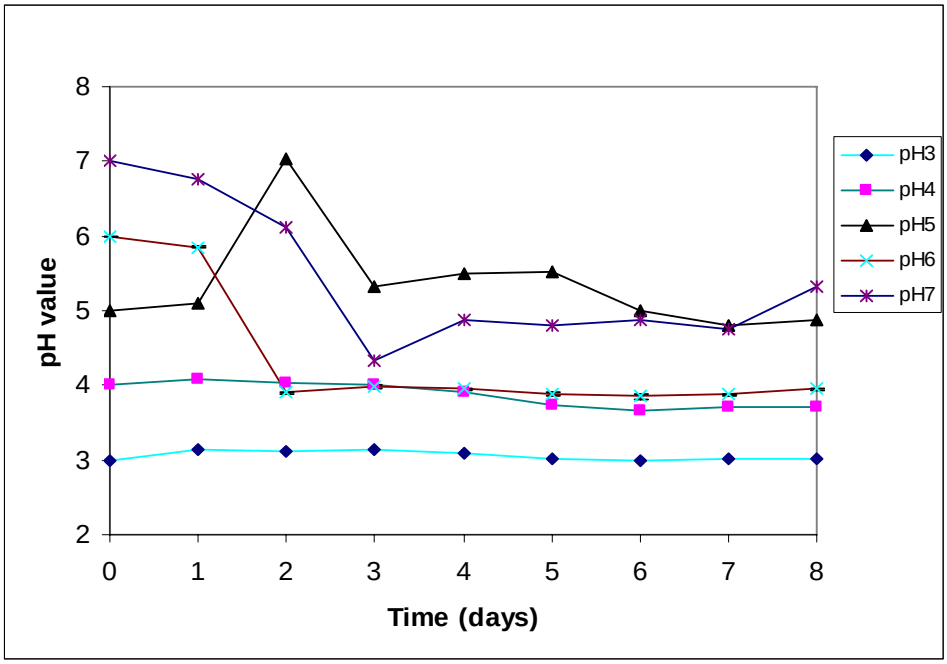


Figure 3.8 Change in pH for Orange II decolorization cultures with different initial pH values

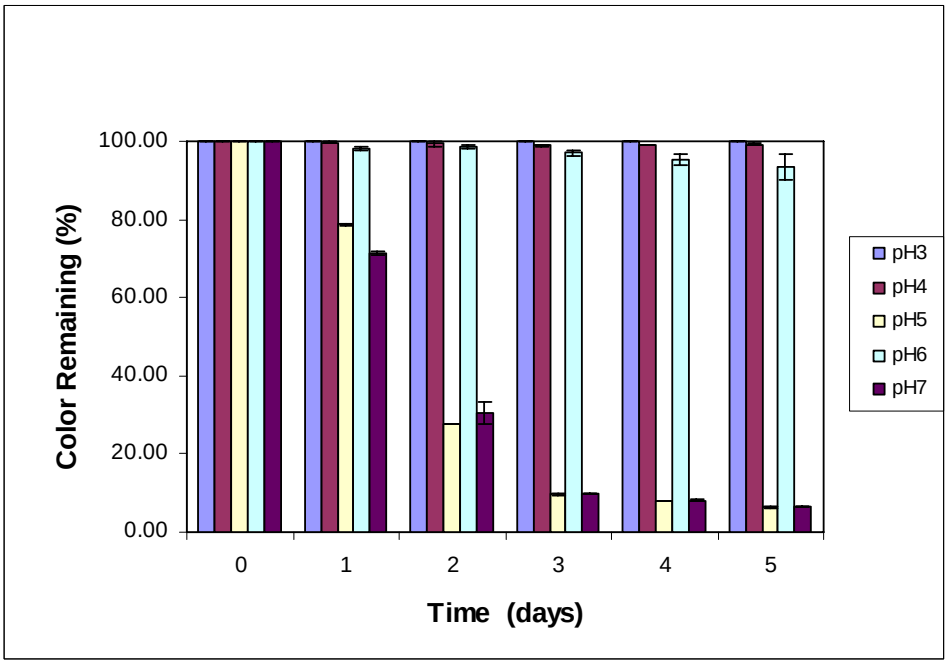


Figure 3.9 Effect of initial pH on Orange II decolorization

The results for Acid Red 29 were very similar to those of Orange II except for pH 6. ANOVA analysis gave a P-value of 3.67×10^{-3} for 3 day decolorization results, which indicated that initial pH can significantly affect the decolorization rate (refer to Appendices). In the experiments for Acid Red 29 (Figure 3.10 and 3.11), cultures with initial pH at 5, 6 and 7 all achieved more than 80 percent decolorization after 5 days. Once again, cultures having initial pH at 5 and 7 self-stabilized their pH after the addition of dye. Cultures with an initial pH 6 were not able to reach close to 5 until 5 days after addition of dyes, but a high degree of decolorization was obtained. This was assumed to be partially due to the structural difference between Orange II and Acid Red 29. However, this assumption has not yet been proved by experiment. The pH 4 cultures showed about 25 percent color removal after 5 days, which may due to bioadsorption of dyes onto fungal mass as in the case of cultures having an initial pH at 5 (discussed later in section 3.5).

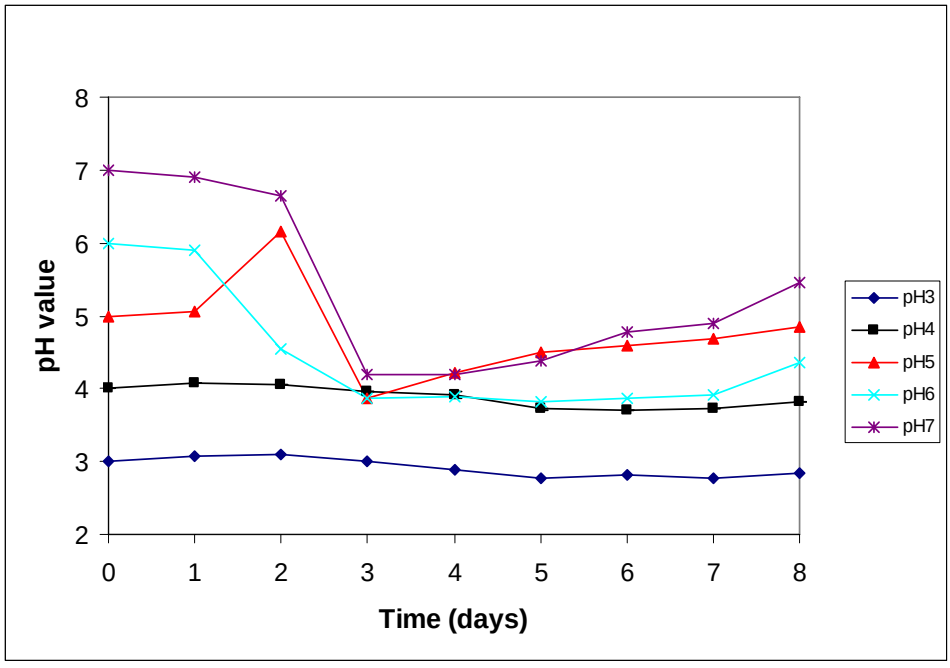


Figure 3.10 Change in pH for Acid Red 29 decolorization cultures with different initial pH values

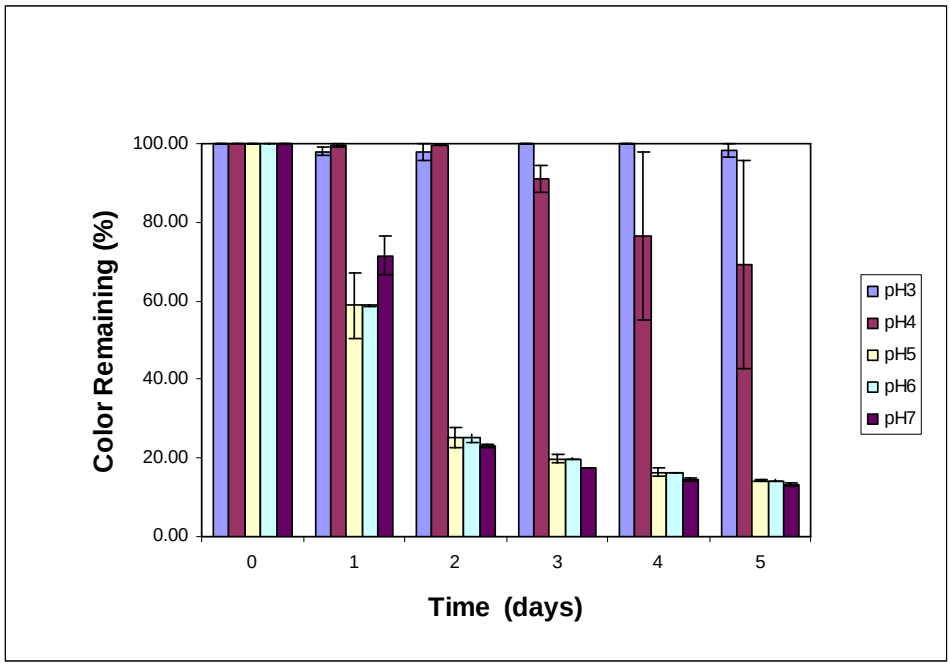


Figure 3.11 Effect of initial pH on Acid Red 29 decolorization

Similar to the results obtained for Acid Red 29, cultures having initial pH's 5, 6 and 7 all decolorized Remazol Brilliant Blue R (RBBR) to a considerable degrees (Figure 3.12 and 3.13), but not to the extent as for Orange II and Acid Red 29. ANOVA analysis gave a P-value of 1.64×10^{-7} for 3 day decolorization results, which indicated that the initial pH played a significant role in decolorization rate (refer to Appendices).

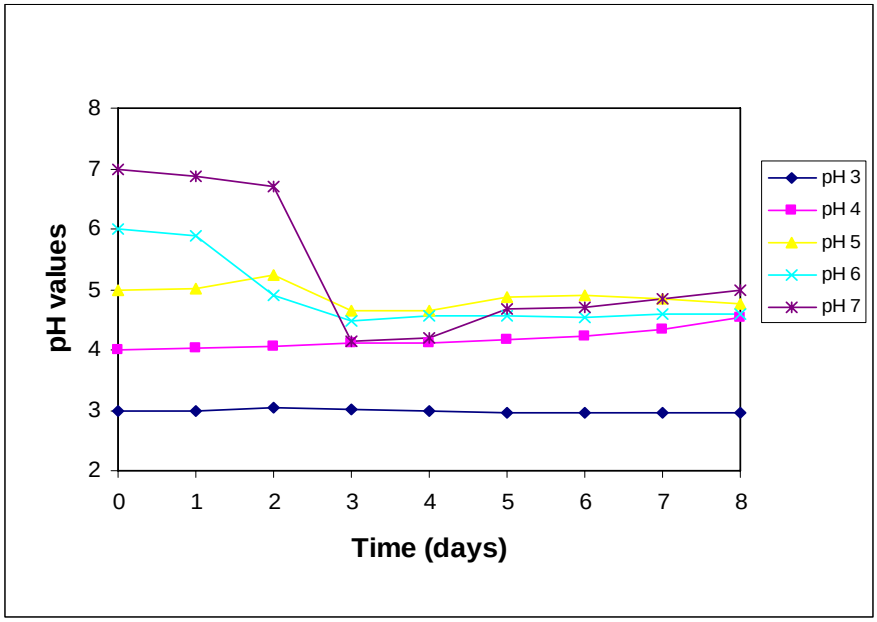


Figure 3.12 Change in pH for RBBR decolorization cultures with different initial pH values

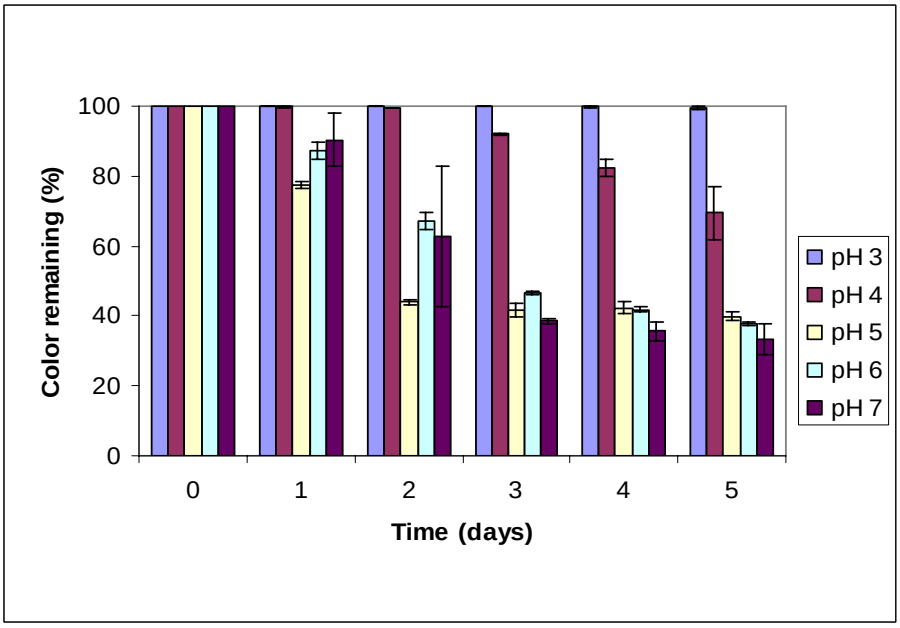


Figure 3.13 Effect of initial pH on RBBR decolorization

As previously addressed, the proper pH in the fungal cultures is essential to achieve a satisfactory decolorzation result. Both buffer concentration and initial pH in the culture can significantly affect the pH during incubation and decolorization. Therefore, research is needed to find the optimal buffer concentration and optimal initial pH for a fungus concerning specific applications.

For the decolorization of Orange II, Acid Red 27 and Remazol Brilliant Blue R by *Pleurotus ostreatus*, the appropriate working pH appeared to be 5 or 7. The pH 5 was chosen over 7 since most of the previous work done by our research group was based on pH 5. Ten mM or 20 Mm sodium acetate buffer is the appropriate buffer for the decolorization of these three dyes. Buffer at 10mM was chosen over 20mM for the later study of five azo dyes since it gave better decolorization rate for Acid Red 29 but not for RBBR, which has a much less structural similarity to the five azo dyes to be studied. The complete answers to why and how buffer concentration and initial pH affect the enzymology of fungi during decolorization cannot be given at this point, and more research is needed.

3.3 The effect of agitation on decolorization.

Fungal cultures incubation can be done either statically or under agitation. Depending upon the fungi species, enzymes and the dyes, agitation may or may not promote decolorization efficiency. Kim et al (1996) has described a decrease in RBBR decolorization in a shaken culture of *Pleurotus ostreatus*. However, Swamy and Ramsay (1999) reported that agitation was crucial for decolorization of several dyes by *Bjerkandera* sp. BOS55, *Phanerochaete chrysosporium* and *Trametes versicolor*. Jarosz et al (2002) also showed that shaken cultures gave better decolorization results than static ones. Ha et al (2001) investigated the effect of

agitation on fungal pellets growth and production of MnP and laccase. It was found that agitation was essential to produce MnP, because of the need for increased oxygen concentration in the culture. However, agitation did not influence the laccase production very much. In a study of decolorization of Orange G and RBBR by three white rot fungi, *Dichomitus squalens, Ishchnoderma resinosum* and *Pleurotus calyptratus*, the effect of agitation on decolorization was found to depend not only on the fungal species and enzymes, but also on dye types (Eichlerova et al, 2005). *Dichomitus squalens* showed a comparable decolorization rate under both shaken and static conditions in spite of the fact that apparent different levels of MnP and laccase were produced. *Ishchnoderma resinosum* and *Pleurotus calyptratus* produced much more laccase under static condition than under shaken conditions, while the production of MnP was not affected. However, static cultures of *Ishchnoderma resinosum* and *Pleurotus calyptratus* gave better decolorization than shaken cultures for Orange G but not for RBBR. Therefore, the effect of agitation on decolorization cannot be simply categorized as increasing or decreasing production of certain types of enzymes.

In our study, the cultures were incubated and shaken at 150 rpm for 3 days to achieve the formation of uniform pellets. This formation is considered to be very important for enzyme production from *Pleurotus ostreatus* for (Ha et al, 2001). Dye stock solutions were then added. In first three days of decolorization, cultures were kept static in a temperature chamber. Color was measured after the cultures were taken out of the chamber and shaken at 150 rpm for 10 minutes. As can be seen, almost no color was removed for all three dyes during the static period. After three static days, shaking was started, and decolorization was observed subsequently (Figure 3.14). After 3 days of agitation, more than 90 percent of Orange II was decolorized. Decolorization was 70 and 60 percent, respectively, for Acid Red 29 and Remazol Brilliant Blue

R. These results showed that agitation was essential for the decolorization of these three dyes. ANOVA analysis of 3rd decolorization data without and after agitation gave values much smaller than 0.05 for all three dyes, which indicated that agitation was significantly important for decolorization of these three water soluble dyes (refer to Appendices). The first three days of static conditions may or may not give a higher level of enzymes production. However, without agitation no reactions to break down dye molecules took place. The decolorization after agitation began may due to the improved mass transfer and increased oxygen concentration in cultures (Ha et al, 2001, Swamy and Ramsay, 1999). A study on *Phanerochaete chrysosporium* indicated that during lignin degradation the lignin has to bind to the fungal wall for effective degradation (Kirk and Farrell, 1987), and that agitation can promote such binding. If decolorization of dyes also requires such binding, agitation can likely give better decolorization efficiency.

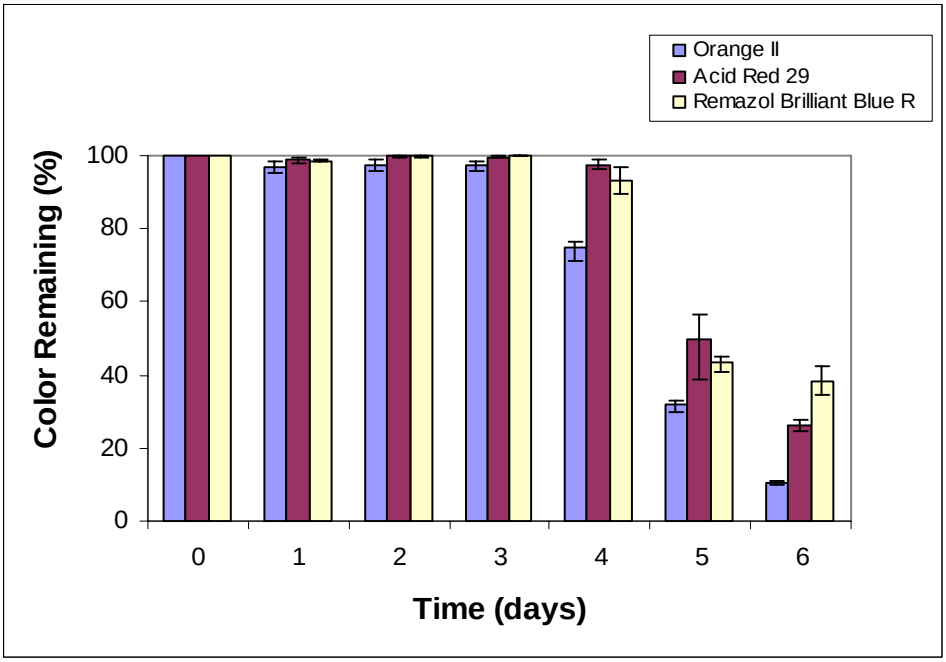


Figure 3.14 Effect of agitation (150 rpm) on decolorization

3.4 The effect of incubation temperature on decolorization of Orange II by *Pleurotus ostreatus*

Besides buffer concentration and pH of the culture, the incubation temperature can also apparently influence the growth of fungal culture, therefore affect the decolorization result. Orange II was used to test how incubation temperature may affect the decolorization. Fungal cultures were incubated under 3 different temperatures, 25 ºC, 30 ºC and 35ºC. After 3 days incubation, stock solutions of Orange II were added to cultures to give a 100 ppm initial concentration. Decolorization was monitored daily until the fifth day. At all three temperatures,

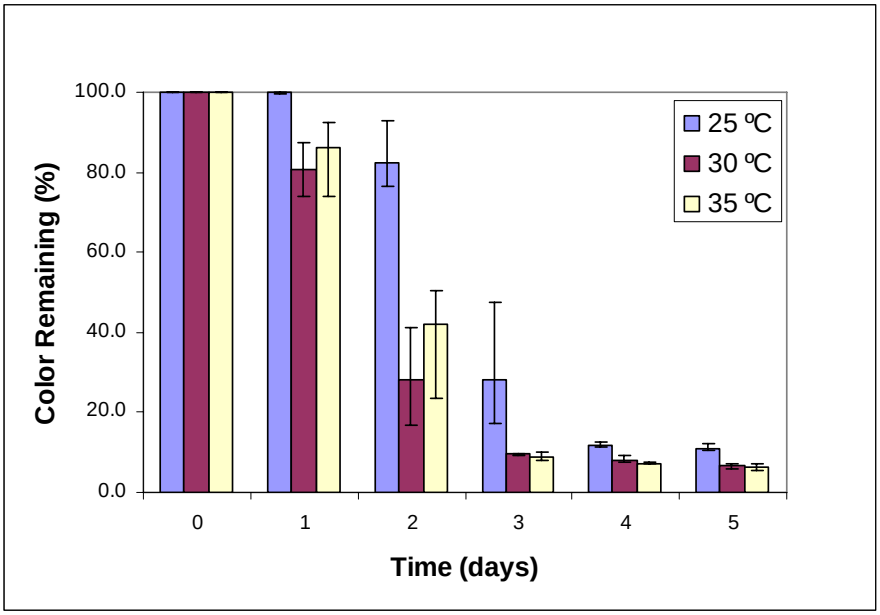


Figure 3.15 Effect of temperature on Orange II decolorization

substantial decolorization was observed after 3 days (Figure 3.15). The 25 ºC samples showed a slower rate of decolorization than 30 ºC and 35 ºC. After 2 days of decolorization, only 20 percent of color was removed at 25 ºC, while more than 50 percent of color was removed at 30ºC and 35ºC.

Temperature was shown to have significant influence on fungal decolorization with a P-value of 0.004995 from ANOVA analysis. Temperatures at 30 ºC and 35 ºC appeared to give faster decolorization than 25 ºC. However, when future application is considered, 25 ºC may be preferred because of energy concerns.

3.5 Bioadsorption of three water soluble dyes onto the fungal mass.

In the decolorization of textile dyes by white rot fungi, removal of dye from cultures is not only due to the actual break-down of dye molecules but also due to the bio-adsorption of dye by the fungal mass. In order to determine if the high degrees of decolorization previously observed were caused by bioadsorption, cultures (incubated with 10 mM buffer and at pH 5) were autoclaved, and dyes were added to see how much adsorption occurred with dead fungal pellets. The results are shown in Table 3.4.

Table 3.4 Bioadsorption of three water soluble dyes on fungal mass

Dyes	Color Remaining (%)		
	After 1 day	After 2 days	After 3 days
Orange II	79.6 ± 0.4	79.5 ± 1.4	78.3 ± 1.6
Acid Red 29	96.2 ± 2.2	95.8 ± 2.3	95.7 ± 2.7
RBBR	90.5 ± 5.7	90.6 ± 5.8	90.8 ± 5.7

As is shown, approximately 20 percent of Orange II was absorbed by the dead fungal mass within 24 hours of agitation at 150 rpm. This amount of bioadsorption of Orange II by the dead fungal mass could contribute to the first day decolorization in a live fungal culture, which was also about 20 percent. However, extending the agitation time to 48 or 72 hours did not show further bioadsorption. This indicates that the significant decolorization seen in live cultures was primarily due to the actual break-down of dye molecules by the fungus. Only 5 percent of the color of Acid Red 29 was removed because of bioadsorption. The longer time did not increase

the bioadsorption. Thus, almost all of the decolorization in cultures having Acid Red 29 was primarily due to the fungus' ability to degrade dye molecules. RBBR showed a higher level of bioadsorption degree than Acid Red 29 but lower than Orange II, and about 10 percent of the dye was adsorbed by the dead fungal mass. As was shown earlier, more than 50 percent of RBBR was decolorized in a live fungal culture after 3 days; therefore the great amount of the decolorization of RBBR was due to fungal enzymatic degradation of dye.

CHAPTER 4

ANALYSIS OF DEGRADATION OF NITROPHENYLAZOPHENYLAMINE DYES BY WHITE ROT FUNGUS *PLEUROTUS OSTREATUS*

Zhao and Hardin (2006) have developed an analytical profile for analysis of degradation of azo dyes by the white rot fungus *Pleurotus ostreatus*. However, questions still remain as to whether a dye structurally similar to Disperse Orange 3 will give similar primary degradation products (Zhao, 2006), and whether further degradation of the primary products will occur. Five nitrophenylazophenylamine dyes, Disperse Red 1, Disperse Red 19, Disperse Orange 1, Disperse Orange 25 and Disperse Red 13 were studied for their degradation by *Pleurotus ostreatus.* The possible degradations of the primary products were also studied.

4.1 Primary degradation of hydrophobic azo dyes by white rot fungus *Pleurotus ostreatus*

Disperse Red 1 differs from Disperse Orange 3 only in one end of the molecular structure; therefore, it is quite possible for Disperse Red 1 to produce the same primary products as those from Disperse Orange 3. The gas chromatogram showed that the three major products from Disperse Orange 3, nitrobenzene, 4-nitrophenol and 4-nitroaniline, were also produced from Disperse Red 1 in the presence of *Pleurotus ostreatus*. After 1 day, only veratryl alcohol was detected in the culture (Figure 4.1). Veratryl alcohol is produced in the secondary metabolism cycle of *Pleurotus ostreatus,* and it is essential in the lignin peroxidase cycle during oxidations of azo dyes (Paszcznski and Crawford, 1991). However, *Pleurotus ostreatus* is believed not to produce any LiP (Wesenberg et al, 2003). Veratryl alcohol can also be oxidized

by both Versatile peroxidase (Heinfling et al., 1998b) and veratryl alcohol oxidase (VAO) (Sannia et al., 1991) to veratryl aldehyde. The occurrence of veratryl alcohol, therefore did not necessarily indicate the existence of LiP.

Nitrobenzene, 4-nitrophenol and 4-nitroaniline were detected in the 4 day degradation culture (Figure 4.2). Furthermore, 4-nitroanisole, a product from 4-nitrophenol through a methylation step, was also detected. Such methylation was reported by Zhao (2006) for *Pleurotus ostreatus*, and methylation of phenolic compounds by another fungus *Phanerochaete chrysosporium*, has also been reported (Valli and Gold, 1991; Valli et al., 1992). The gas chromatogram of the 7 days degradation culture also showed the three primary products and 4-nitroanisole as well (Figure 4.3). These compounds were not detected in the control sample (Figure 4.4).

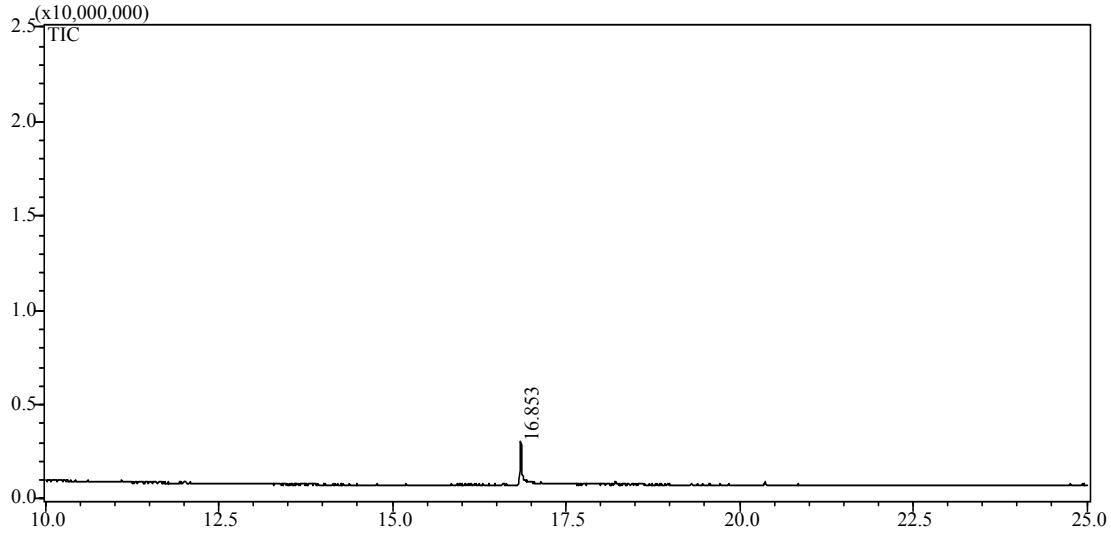


Figure 4.1 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 1 day culture containing Disperse Red 1 — veratryl alcohol (16.853 min)

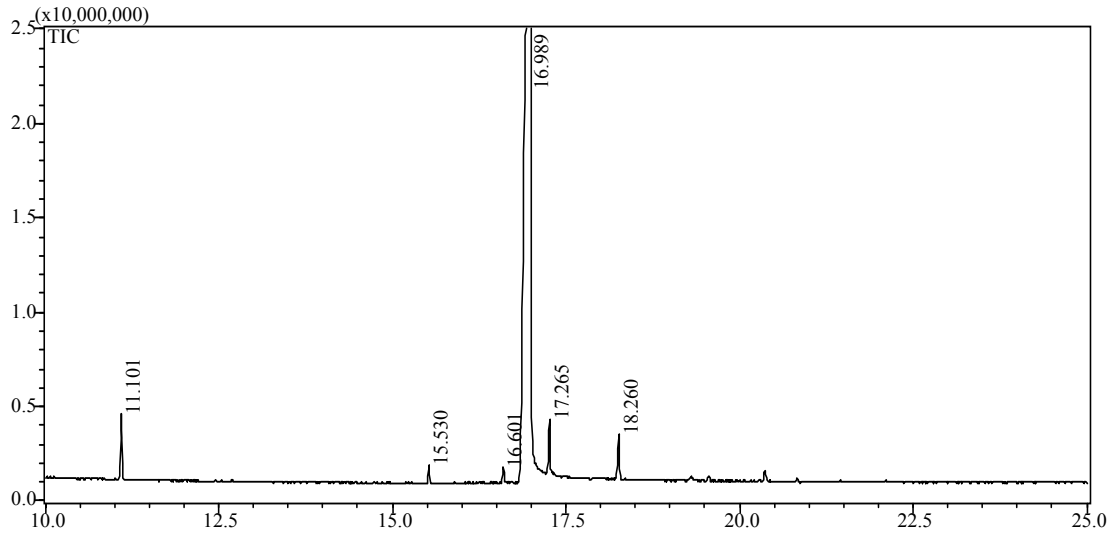


Figure 4.2 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 4 day culture containing Disperse Red 1 — nitrobenzene (11.101 min), 4-nitroanisole (15.530 min), veratryl aldehyde (16.601 min), veratryl alcohol (16.989 min), 4-nitrophenol (17.265 min), 4-nitroaniline (18.260 min).

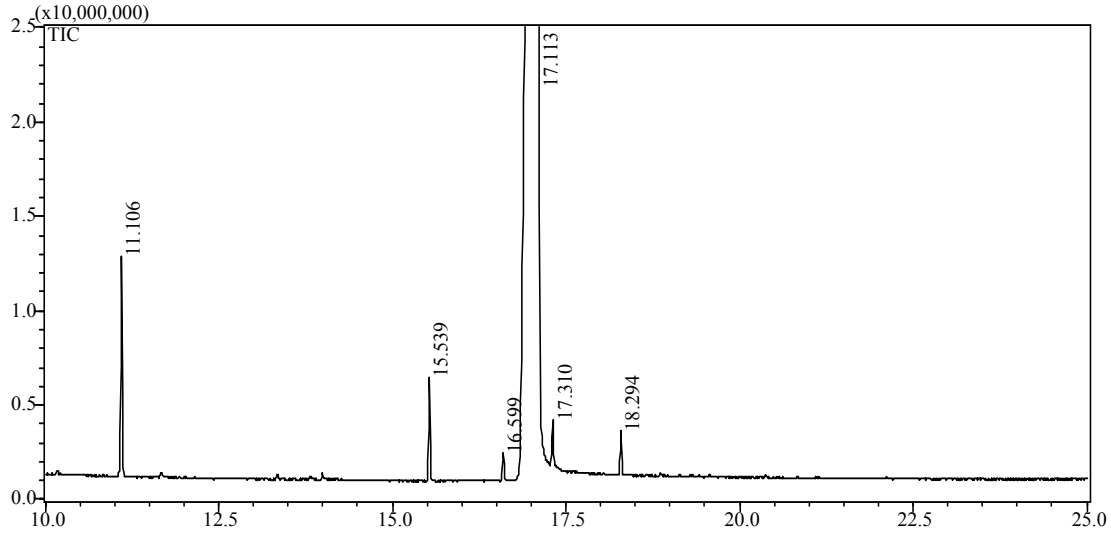


Figure 4.3 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 7 day culture having Disperse Red 1 — nitrobenzene (11.106 min), 4-nitroanisole (15.539 min), veratryl aldehyde (16.599 min), veratryl alcohol (17.113 min), 4-nitrophenol (17.310 min), 4-nitroaniline (18.294 min)

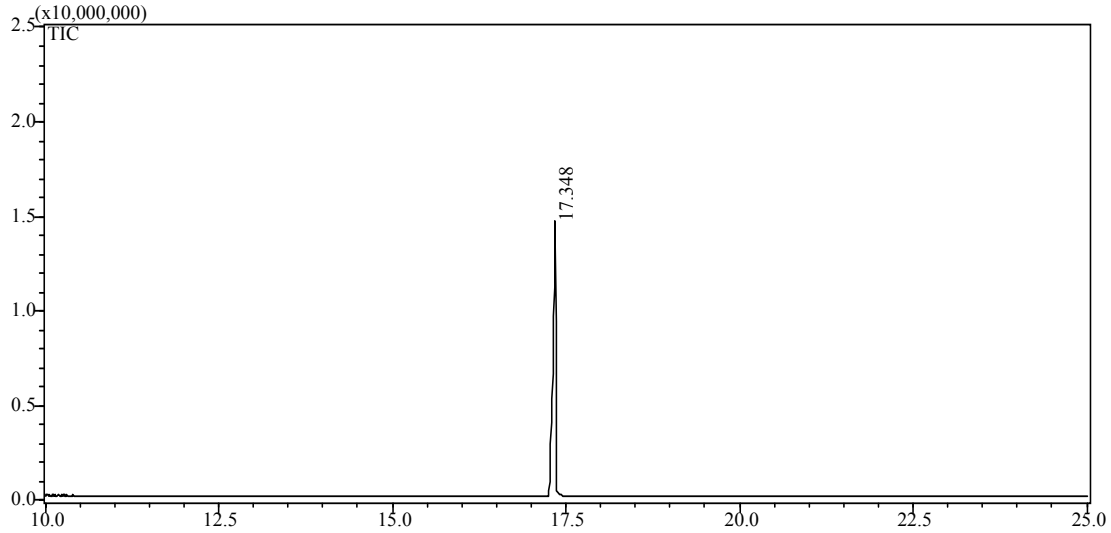


Figure 4.4 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 7 day pure culture — veratryl alcohol (17.348 min)

The 7 day degradation sample was also analyzed by HPLC; the signal was monitored at 5 different wavelengths, the maximum absorption wavelengths for the possible products. The results also confirmed that the same three products were produced from Disperse Red 1 and Disperse Orange 3 (Figure 4.5), and they were not detected in the pure culture (Figure 4.6). It is believed that both nitrobenzene and 4-nitrophenol can be generated from the unstable intermediate, 4-nitrophenyldiazene, which can then form a free radical at the para-position to the nitro group on the benzene ring by loss of nitrogen. This free radical can further accept a hydrogen free radical or a hydroxyl free radical to form nitrobenzene or 4-nitrophenol (Zhao, Ph.D dissertation, 2004). Figure 4.5 shows that another new compound that was found in the 7 day degradation culture; it was 1,2-dimethoxy-4-nitrobenzene (retention time = 29.385 min, 344 nm). A later study of the degradation of 4-nitrophenol showed that 1,2-dimethoxy-4-nitrobenzene is a possible product from 4-nitrophenol.

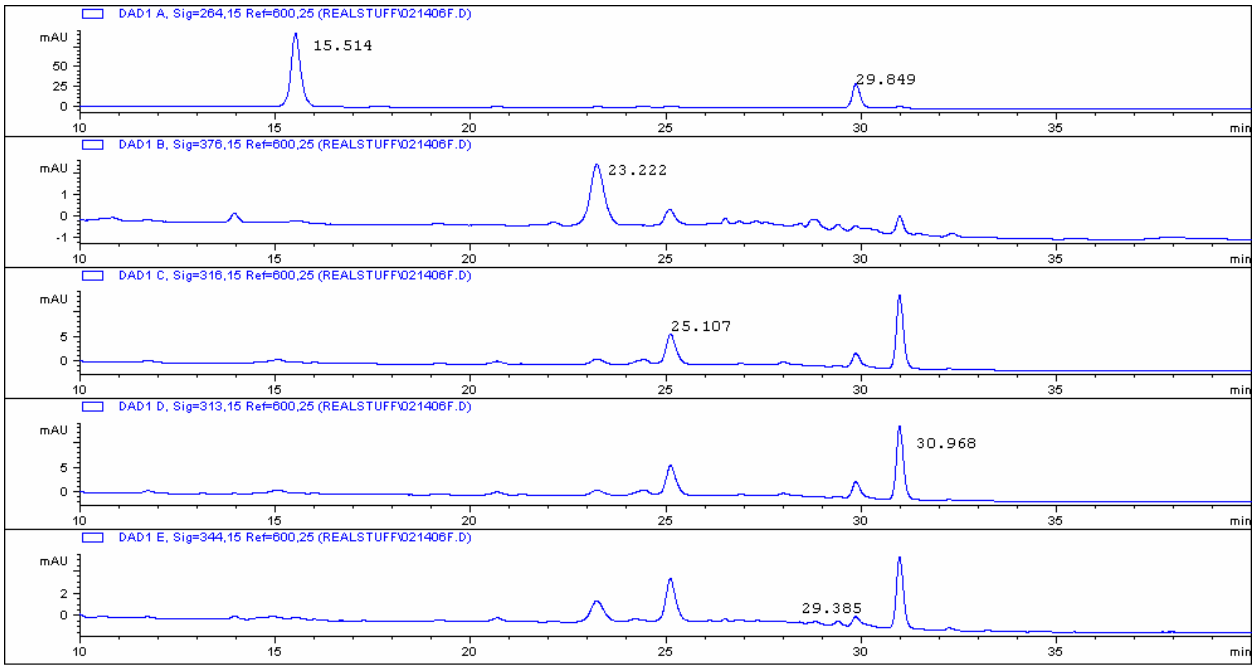


Figure 4.5 HPLC chromatogram of 7 day culture having Disperse Red 1 — veratryl alcohol (15.514 min, 264 nm), 4-nitroaniline (23.222 min, 376nm), 4-nitrophenol (25.107 min, 316 nm), 1,2-dimethoxy-4-nitrobenzene (29.385 min, 344 nm), nitrobenzene (29.849 min, 264 nm), 4-nitroanisole (30.968 min, 313 nm)

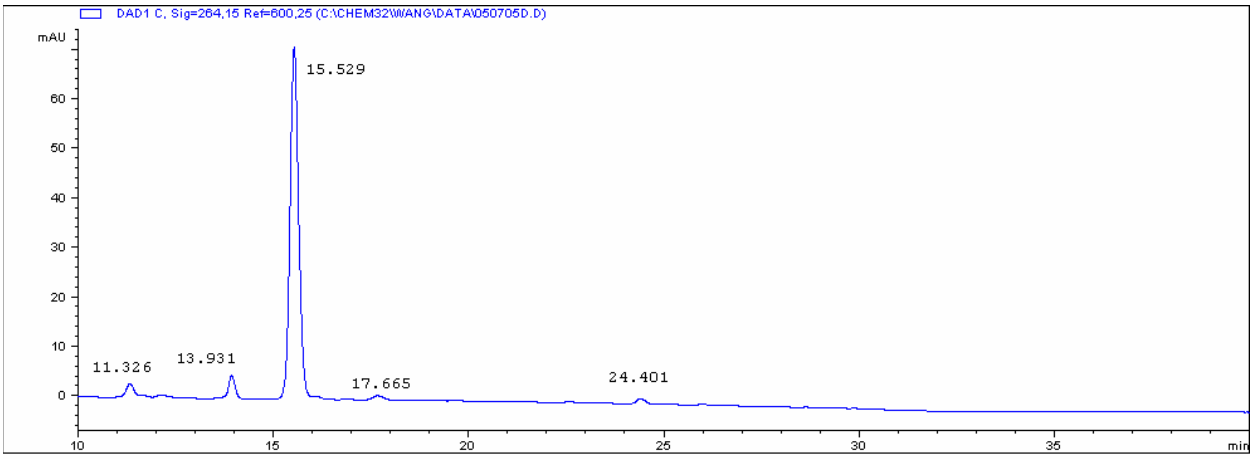


Figure 4.6 HPLC chromatogram of 7 day culture (264 nm) — veratryl alcohol (15.529 min) , veratryl aldehyde (24.401 min), unknown metabolites (11.326 min, 13.931 min and 17.665 min)

Disperse Red 19 has a molecular structure quite similar to that of Disperse Red 1 with a hydrogen replaced by a hydroxyl group at one end of the molecule. Since Disperse Red 1 can produce nitrobenzene, 4-nitrophenol and 4-nitroaniline in the presence of *Pleurotus ostreatus*, Disperse Red 19 was believed to be able to generate the same 3 major compounds. In the supernatant of the 1 day old culture containing Disperse Red 19, 4-nitroaniline was already detected, while nitrobenzene and 4-nitrophenol were not observed (Figure 4.7). This indicated that the generation of 4-nitroaniline is through a different mechanism than that for production of nitrobenzene and 4-nitrophenol. A reductive cleavage is the most likely mode of production of 4-nitroaniline. Pinheiro et al (2004) reported that the azo bond is vulnerable to reductive cleavage. A schematic degradation pathway of Disperse Orange 3 (Zhao, Ph.D dissertation, 2004) illustrated that a reductive cleavage of azo bond is responsible for formation of 4-nitroaniline, while nitrobenzene and 4-nitrophenol are likely products from the unstable intermediate, 4-nitrophenyldiazene. Chromatograms of the 4 and 7 day cultures showed the formation of nitrobenzene and 4-nitrophenol, along with 4-nitroanisole (Figure 4.8 and 4.9). Several unknown metabolites were detected in 7 the day culture. They were either seen in the pure culture or unable to be identified for clear structure information. The HPLC results are well in accordance with the GC/MS results, and 1,2-dimethoxy-4-nitrobenzene was again detected (Figure 4.10).

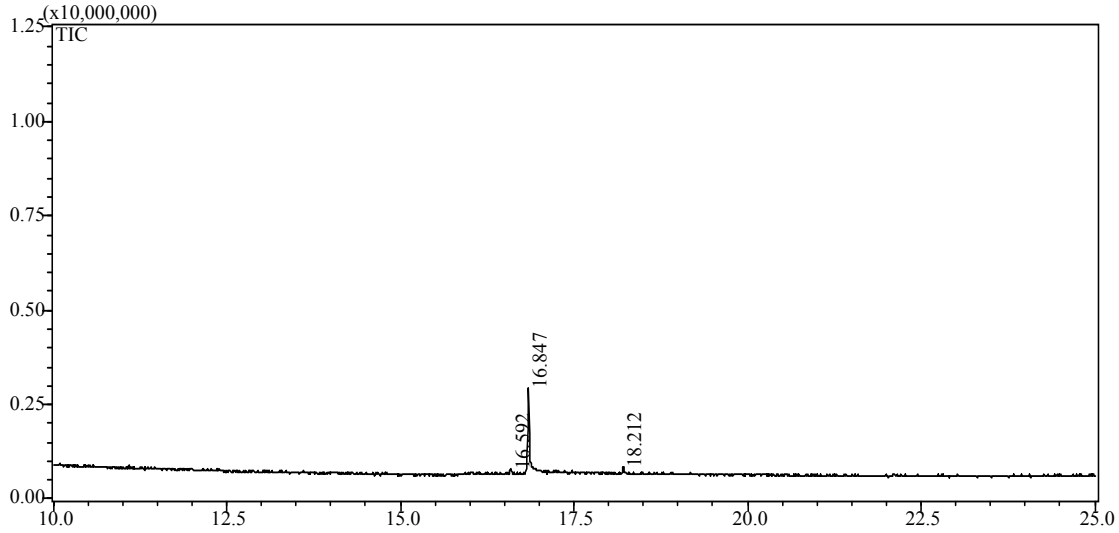


Figure 4.7 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 1 day culture containing Disperse Red 19 — veratryl aldehyde (16.592 min), veratryl alcohol (16.847 min), 4-nitroaniline (18.219 min)

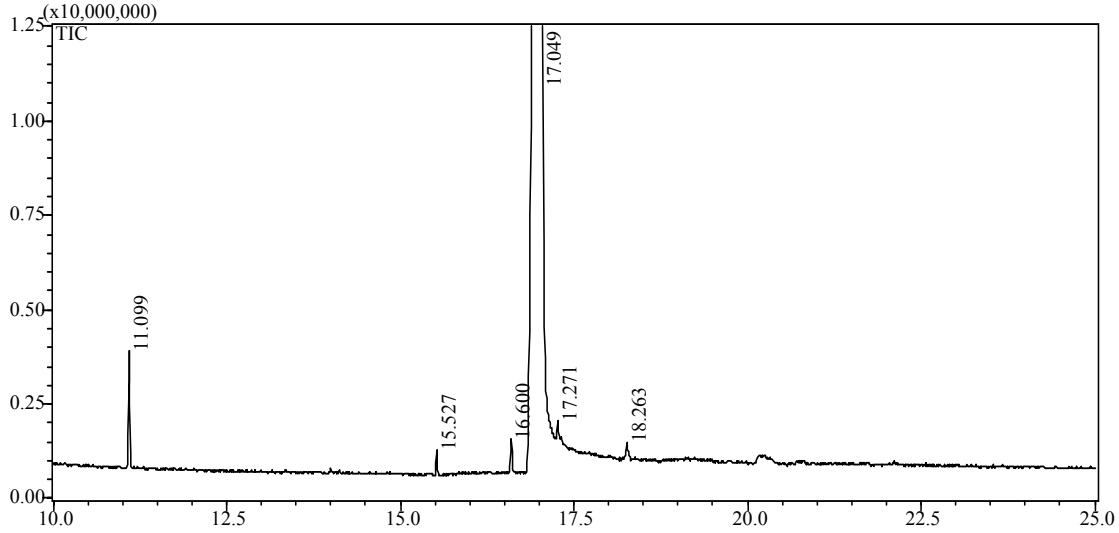


Figure 4.8 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 4 day culture containing Disperse Red 19 — nitrobenzene(11.099min), 4-nitroanisole(15.527min), veratryl aldehyde (16.600min), veratryl alcohol (17.049 min), 4-nitrophenol(17.271min), 4-nitroaniline(18.263min)

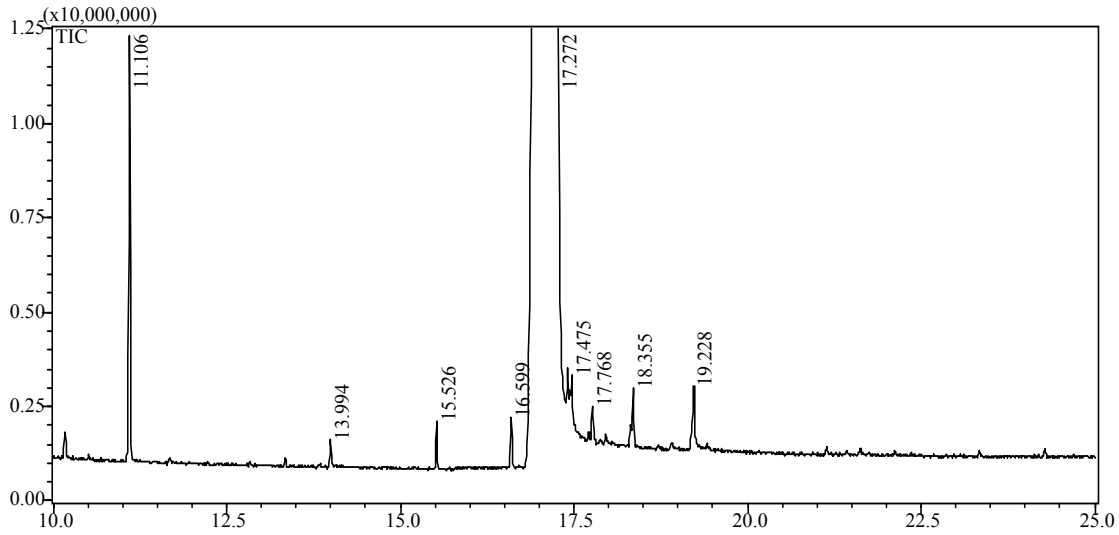


Figure 4.9 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 7 day culture containing Disperse Red 19 — nitrobenzene (11.106 min), 4-nitroanisole(15.526 min), veratryl aldehyde (16.599 min), veratryl alcohol (17.272 min), 4-nitrophenol(17.475 min), 4-nitroaniline(18.355 min). Unknown metabolites (13.994 min, 17.768 min, 19.228 min)

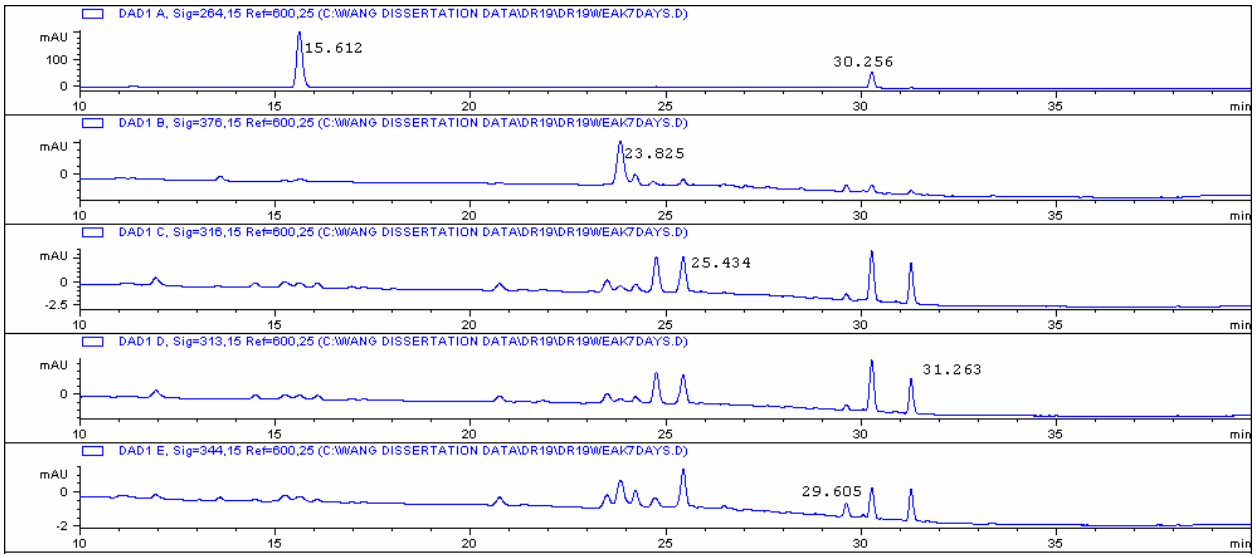


Figure 4.10 HPLC chromatogram of 7 day culture containing Disperse Red 19 — veratryl alcohol (15.612 min, 264 nm), 4-nitroaniline (23.825 min, 376 nm), 4-nitrophenol (25.434 min, 316 nm), 1,2-dimethoxy-4-nitrobenzene (29.605 min, 344 nm), nitrobenzene (30.256 min, 264 nm), 4-nitroanisole (31.263 min, 316 nm)

Given the results of Disperse Red 1 and Disperse Red 19, it is concluded that an azo dye having its partial structure the same as the structure shown in Figure 4.11 is quite likely to be capable of the production of three major products in the presence of *Pleurotus ostreatus*.

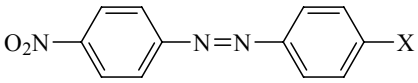


Figure 4.11 A structure shared by the five hydrophobic azo dyes being studied in this research

Disperse Orange 1 and Disperse Orange 25 both fall into the group of azo dyes having such a structure in their molecules. Therefore, it is not unreasonable to predict that nitrobenzene, 4-nitrophenol and 4-nitroaniline will be produced by both of these dyes.

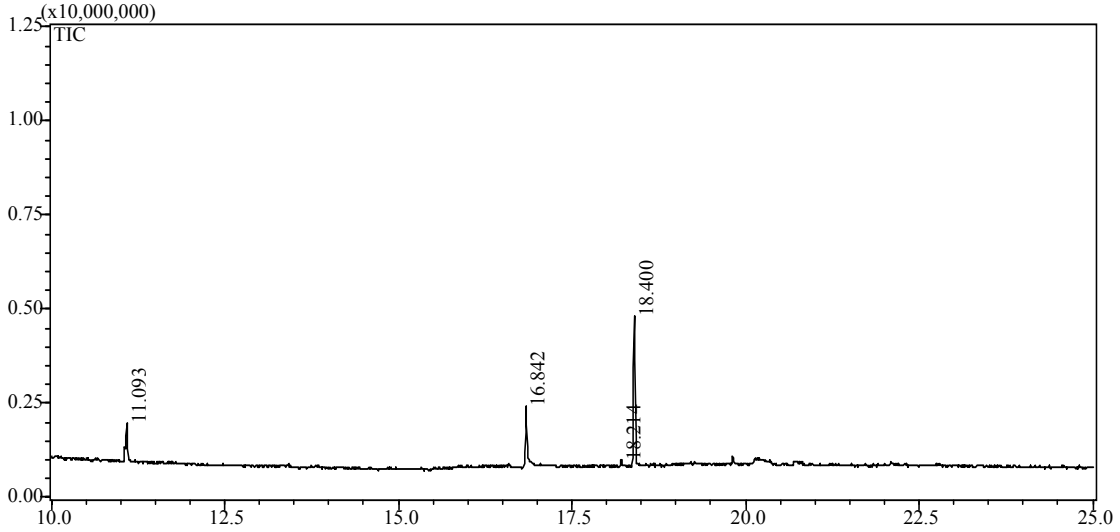


Figure 4.12 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 1 day culture containing Disperse Orange 1— nitrobenzene (11.093 min), veratryl alcohol (16.842 min), 4-nitroaniline (18.214 min), unknown impurity from original dye (18.400 min).

Like Disperse Red 19, the 1 day degradation culture only showed the formation of 4-nitroaniline (Figure 4.12), which once again indicated that the production of 4-nitroaniline was through a different mechanism than that responsible for production of nitrobenzene and 4-nitrophenol. Gas chromatograms of 4 and 7 day cultures clearly illustrated the formation of nitrobenzene and 4-nitrophenol (Figure 4.13 and 4.14). The 4-nitroanisole was only detected in 4 day old culture by GC/MS, but the HPLC chromatogram showed that 4-nitroanisole also existed in the 7 day culture, though in extremely low amount (Figure 4.15).

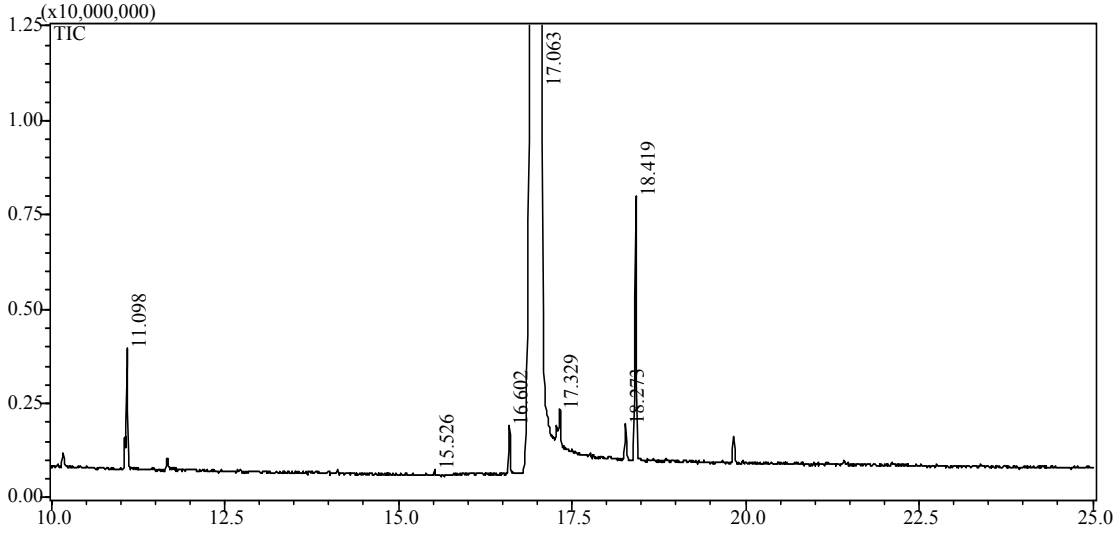


Figure 4.13 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 4 day culture containing Disperse Orange 1 — nitrobenzene (11.098min), 4-nitroanisole (15.526min), veratryl aldehyde (16.602min), veratryl alcohol (17.063 min), 4-nitrophenol (17.329min), 4-nitroaniline (18.273min).

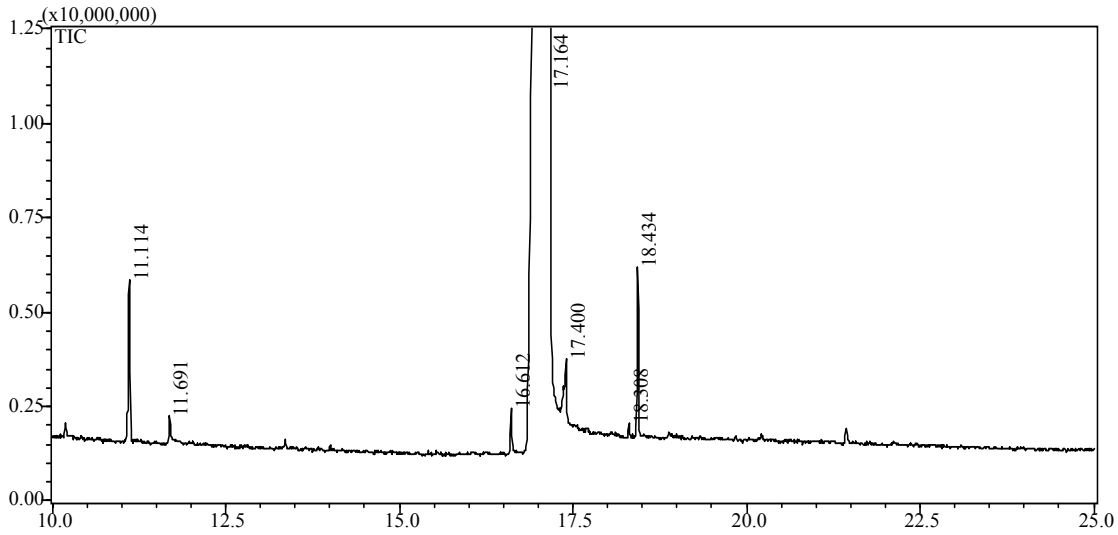


Figure 4.14 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 7 day culture containing Disperse Orange 1 — nitrobenzene (11.114 min), veratryl aldehyde (16.612min), veratryl alcohol (17.164 min), 4-nitrophenol (17.400 min), 4-nitroaniline(18.308 min).

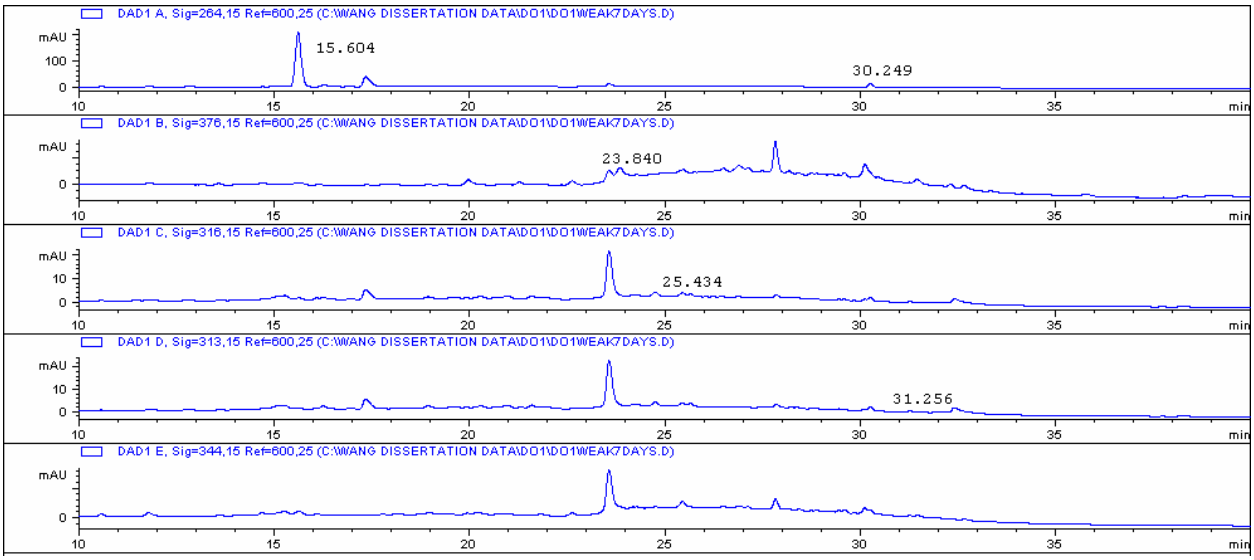


Figure 4.15 HPLC chromatogram of 7 day culture containing Disperse Orange 1 — veratryl alcohol (15.604 min, 264 nm), 4-nitroaniline (23.840 min, 376 nm), 4-nitrophenol (25.434 min, 316 nm), nitrobenzene (30.249 min, 264 nm), 4-nitroanisole (31.256 min, 313 nm).

The 1 day old culture containing Disperse Orange 25 did not show any degradation products beside veratryl alcohol (Figure 4.16). Nitrobenzene was detected in the 4 day culture (Figure 4.17). However, the chromatogram of the 7 day culture (Figure 4.18) showed the formation of three primary products. Like Disperse Orange 1, very little 4-nitroanisole was produced. Nevertheless, the SIC (selected ion chromatogram) display along with the HPLC chromatogram confirmed the production of 4-nitroanisole. SIC can be used to display only the signals from certain ions that are selected by their mass over charge values. Compounds that do not produce the selected ions will not be displayed in the chromatogram. The molecular ion of 4-nitroanisole has a m/z value at 153; therefore m/z 153 was chosen to display the compounds capable of producing such an ion. A peak at 15.522 min was assigned to 4-nitroanisole due to the same mass spectrum and retention time as to standard 4-nitroanisole (Figure 4.19). Another peak at 17.079 min was assigned to veratryl alcohol, which can also generate ions at m/z 153.

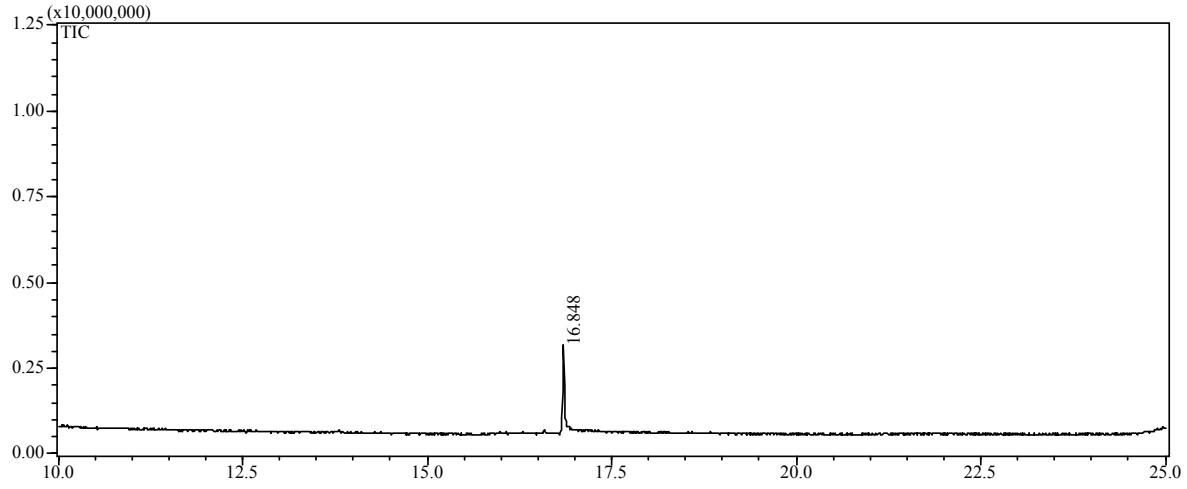


Figure 4.16 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 1 day culture having Disperse Orange 25 — veratryl alcohol (16.848 min).

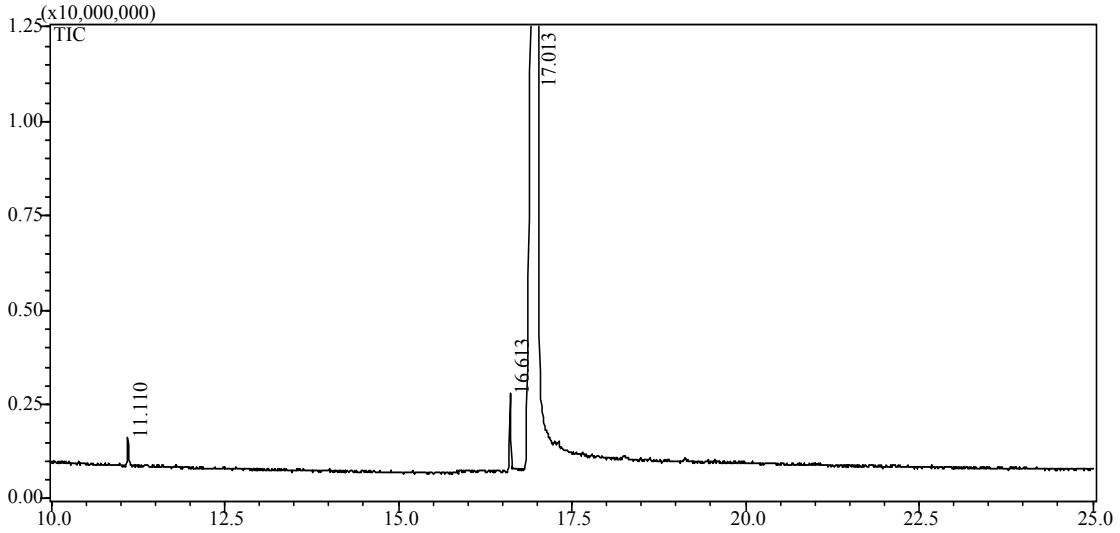


Figure 4.17 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 4 day culture containing Disperse Orange 1 — nitrobenzene (11.110 min), veratryl aldehyde (16.613 min), veratryl alcohol (17.013 min)

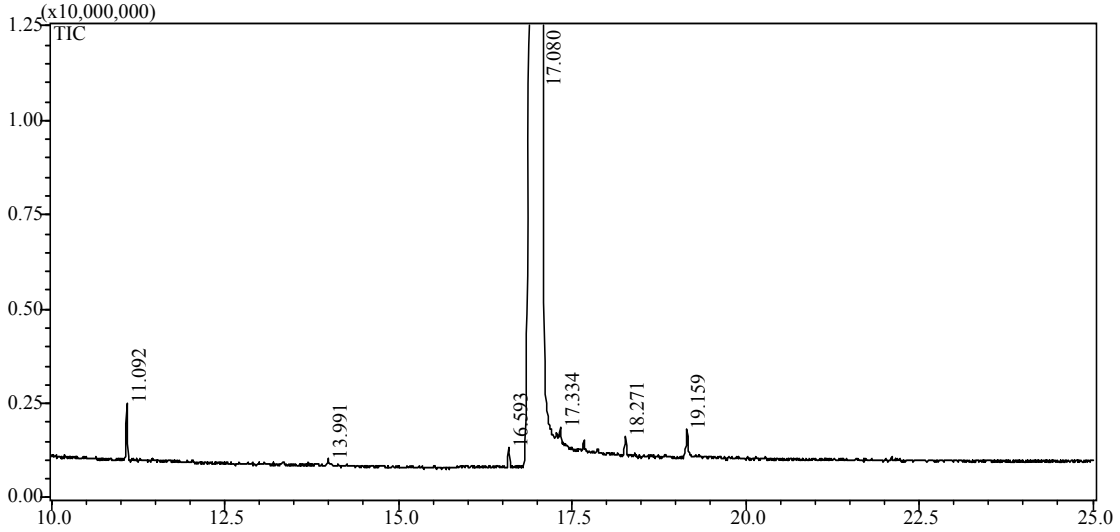


Figure 4.18 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 7 day culture containing Disperse Orange 25 — nitrobenzene (11.092 min), veratryl aldehyde (16.593 min), veratryl alcohol (17.080 min), 4-nitrophenol (17.334 min), 4-nitroaniline (18.271 min), unknown (13.991 min, 19.159 min)

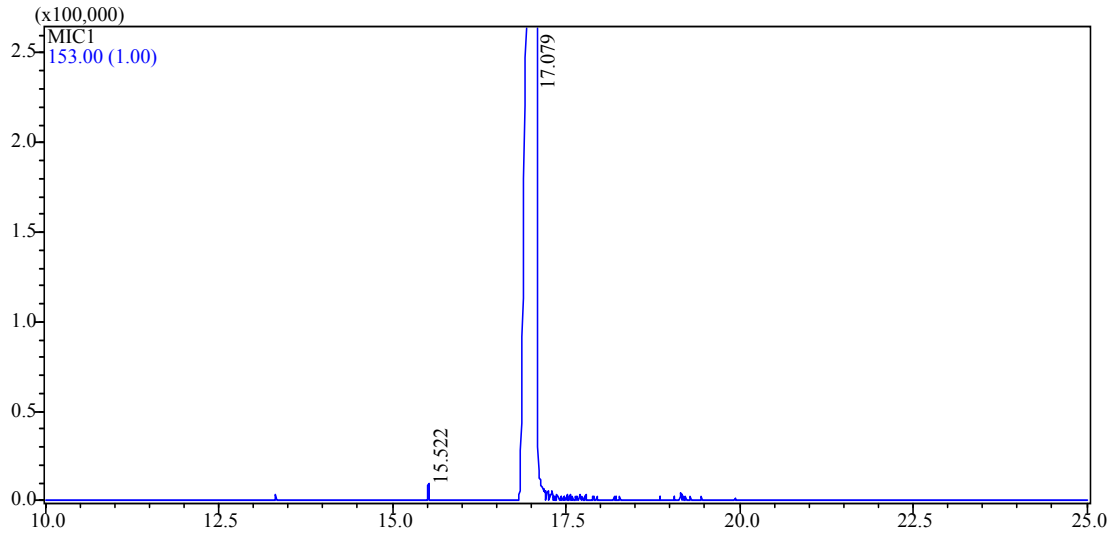


Figure 4.19 SIC display (m/z 153) gas chromatogram of 7 day culture contianing Disperse Orange 25 at m/z 153 — 4-nitroanisole (15.522min) veratryl alcohol (17.079 min)

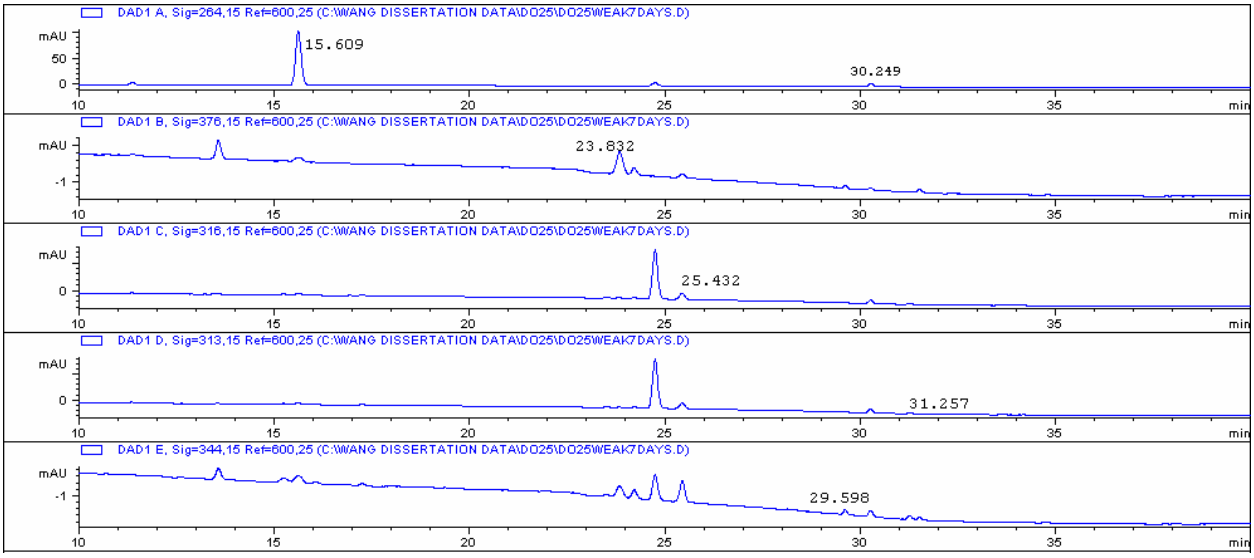
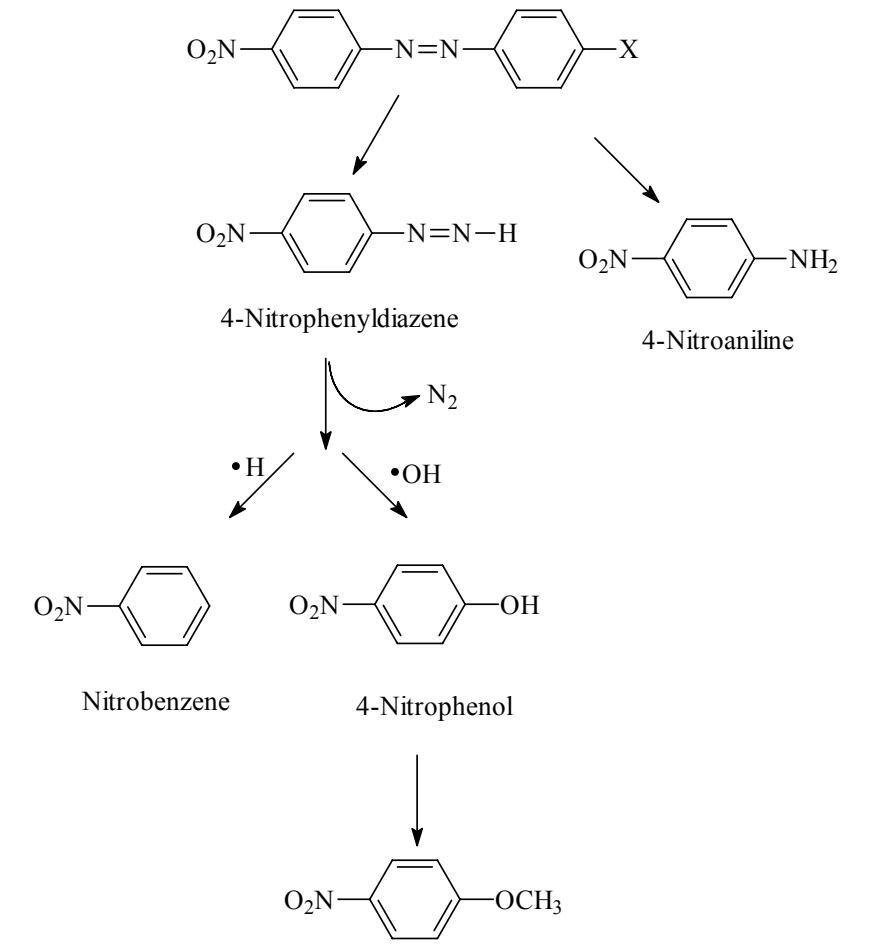


Figure 4.20 HPLC chromatogram of 7 day culture containing Disperse Orange 25 — veratryl alcohol (15.609 min, 264 nm), 4-nitroaniline (23.832min, 376 nm), 4-nitrophenol (25.432 min, 316 nm), 1,2-dimethoxy-4-nitrobenzene (29.598 min, 344 nm), nitrobenzene (30.249 min, 264 nm), 4-nitroanisole(31.257 min, 313 nm).

The HPLC chromatogram (Figure 4.20) of 7 day degradation culture clearly illustrated the production of 4-nitroaniline at 31.257min (313 nm), along with the 3 primary products, nitrobenzene, 4-nitrophenol and 4-nitroaniline. The signal at 344nm showed the production of 1,2-dimethoxy-4- nitrobenzene, which has been previously detected in Disperse Red 1 and Disperse Red 19 degradation cultures. Based on the analysis of degradation of Disperse Red 1, Disperse Red 19, Disperse Orange 1 and Disperse Orange 25, a early stage of degradation pathway of these azo dyes was proposed (Figure 4.21). It is believed that such a pathway can apply to many azo dyes having a structure similar to model compound I. A reductive cleavage can give birth to 4-nitroaniline. The unstable intermediate 4-nitrophenyldiazene eventually transforms into nitrobenzene or 4-nitrophenol, which can further transform into 4-nitroanisole.

Another azo dye, Disperse Red 13 differs from Disperse Red 1 only in the addition of a chloro group at the meta position of the azo bond on the benzene ring having a nitro group. This dye was chosen to see whether the addition of a chloro group would affect the pattern of dye degradation molecule by *Pleurotus ostreatus*. In 1 day degradation culture, only veratryl alcohol was detected, as well as a metabolite at 19.333min whose structure could not be identified (Figure 4.22). The gas chromatograms of both 4 and 7 day degradation cultures illustrated that 1-chloro-3-nitrobenzene and 2-chloro-4-nitroaniline were produced from Disperse Red 13 (Figure 4.23 and 4.24). This indicated that Disperse Red 13 also follows the degradation pattern presented in Figure 4.19. However, if the degradation of Disperse Red 13 was in exact accordance with the pathway shown in Figure 4.21, 2-chloro-4-nitrophenol should have been produced and further produced 2-chloro-4-nitranisole. In fact, 2-chloro-4-nitroanisole was



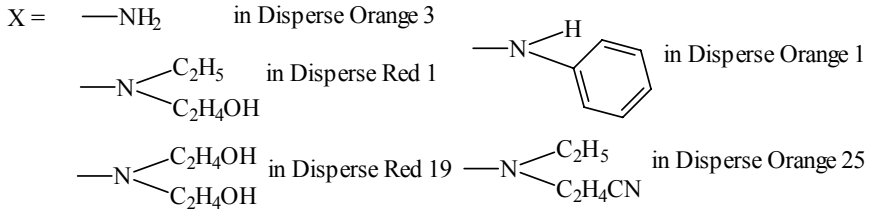


Figure 4.21 The proposed early stage of degradation pathway of several azo dyes by *Pleurotus ostreatus*

Adapted and modified from Zhao's Ph.D dissertation (2006)

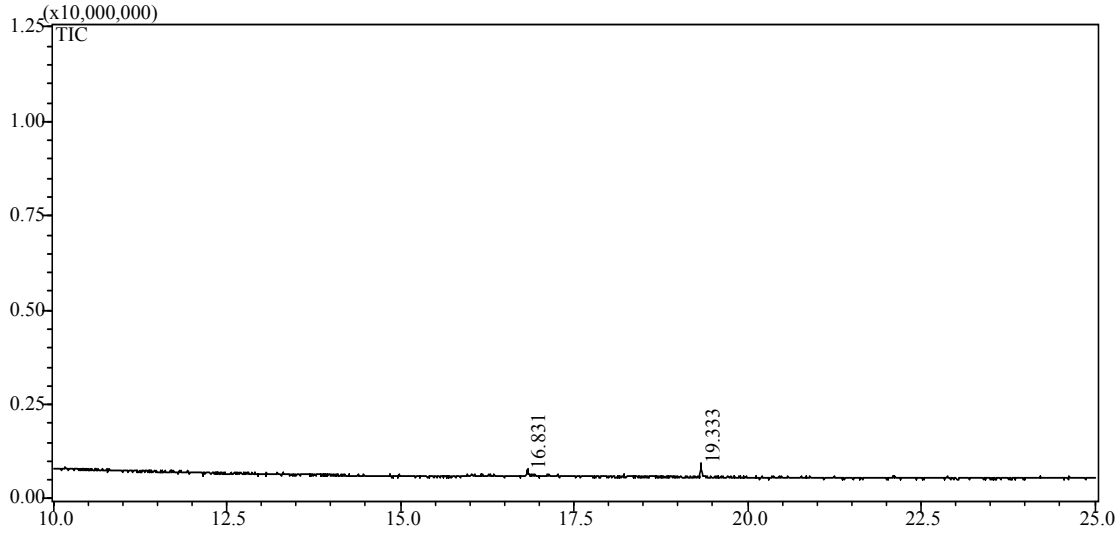


Figure 4.22 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 1 day culture containing Disperse Red 13 — veratryl alcohol (16.831 min), unknown metabolite (19.333min).

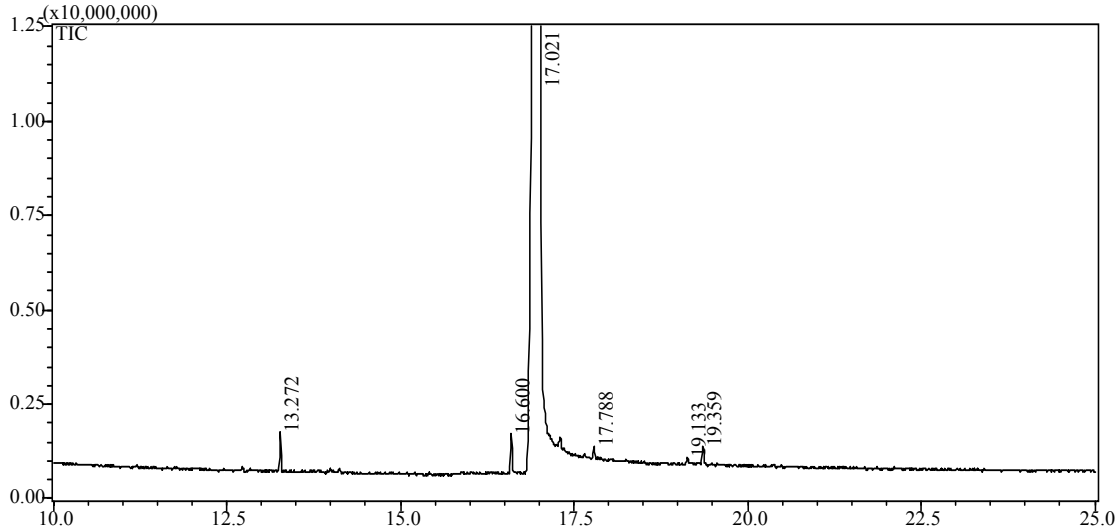


Figure 4.23 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 4 day containing culture containing Disperse Red 13 — 1-chloro-3-nitrobenzene (13.272 min), veratryl aldehyde (16.600 min), veratryl alcohol (17.021 min), 2-chloro-4-nitroanisole (17.788min), 2-chloro-4-nitrooaniline (19.359 min), unknown metabolite (19.133 min).

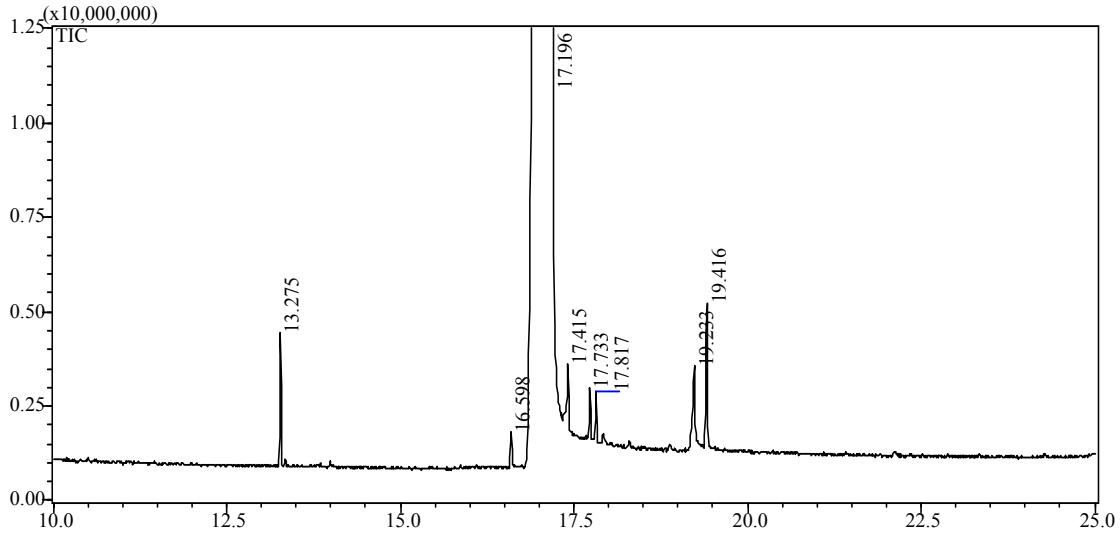


Figure 4.24 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 7 day culture containing Disperse Red 13 — 1-chloro-3-nitrobenzene (13.275min), veratryl aldehyde (16.598 min), veratryl alcohol (17.196 min), 2-chloro-4-nitroanisole (17.817 min), 2-chloro-4-nitrooaniline (19.416 min), unknown metabolites (17.145min, 17.733 min, 19.233 min).

detected in the 4 and 7 day cultures, but these cultures contained no 2-chloro-4-nitrophenol. This indicates that methylation may not be the only mechanism through which nitroanisole was produced. In the transformation of the unstable intermediate 4-nitrophenyldiazene to nitrobenzene and 4-nitrophenol, a key step is for 4-nitrophenyldiazene to lose a nitrogen molecule and form a free radical that is able to accept either hydrogen or hydroxyl free radical to form nitrobenzene or 4-nitrophenol in later reactions (Figure 4.21). Likewise, a corresponding intermediate, 2-chloro-4- nitrophenyldiazene could also occur in the degradation of Disperse Red 13. This compound, like 4-nitrophenyldiazene, can lose a nitrogen molecule to form a free radical and further accept a methoxyl free radical to directly produce 2-chloro-4-nitroanisole. It is suggested that production of 4-nitroanisole in Disperse Red 1 degradation culture may also be due to acceptance of methoxy free radical to the intermediate 4-nitrophenyldiazene rather than merely due to methylation of 4-nitrophenol.

The SIC display at m/z 173 and175 (the molecular ion and A+2 ion) confirmed that 2-chloro-4-nitrophenol was not produced from Disperse Red 13 since the retention time did not correspond to that of 2-chloro-4-nitrophenol (Figure 4.25). The existence of 1,2-dimethoxy-4-nitrobenzene was proved by the signal of ion m/z 183 (the molecular ion) at 18.602 min. This indicated that 4-nitrophenol may not be the only source of the production of this compound. The peak at 29.619 min in HPLC chromatogram at 316 nm also demonstrated the existence of 1,2-dimethoxy-4-nitrobenzene in the degradation culture of Disperse Red 13.

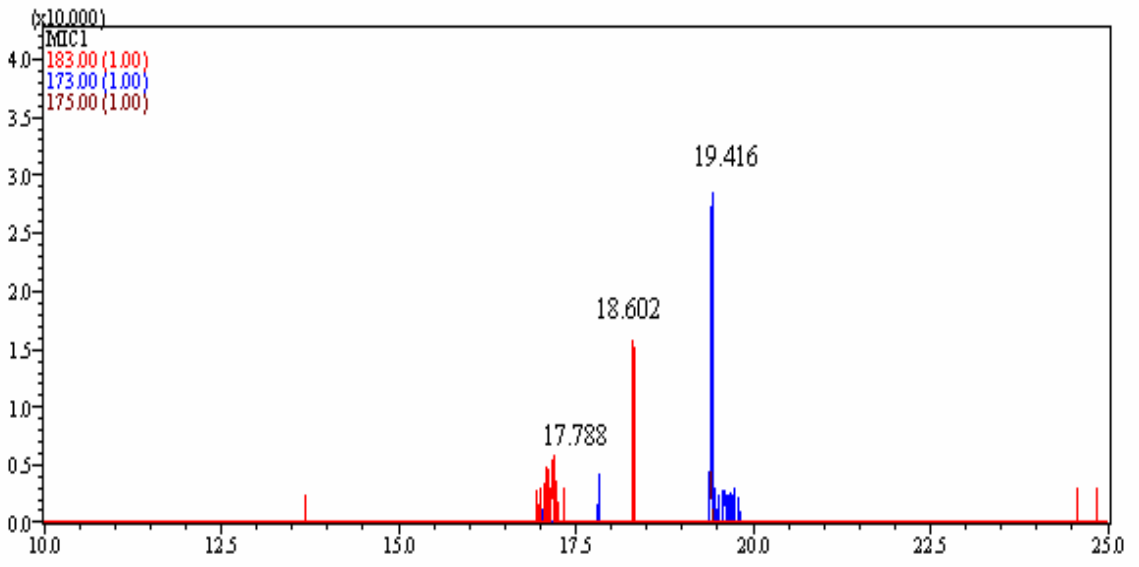


Figure 4.25 SIC display (m/z 173, 175 and 183) of 7 day culture containing Disperse Red 13 — 2-chloro-4-nitroanisole (17.788min, m/z 173), 2-chloro-4-nitroaniline (19.416min, m/z 173), 1,2-dimethoxy-4-nitrobenzene (18.602min, m/z 183).

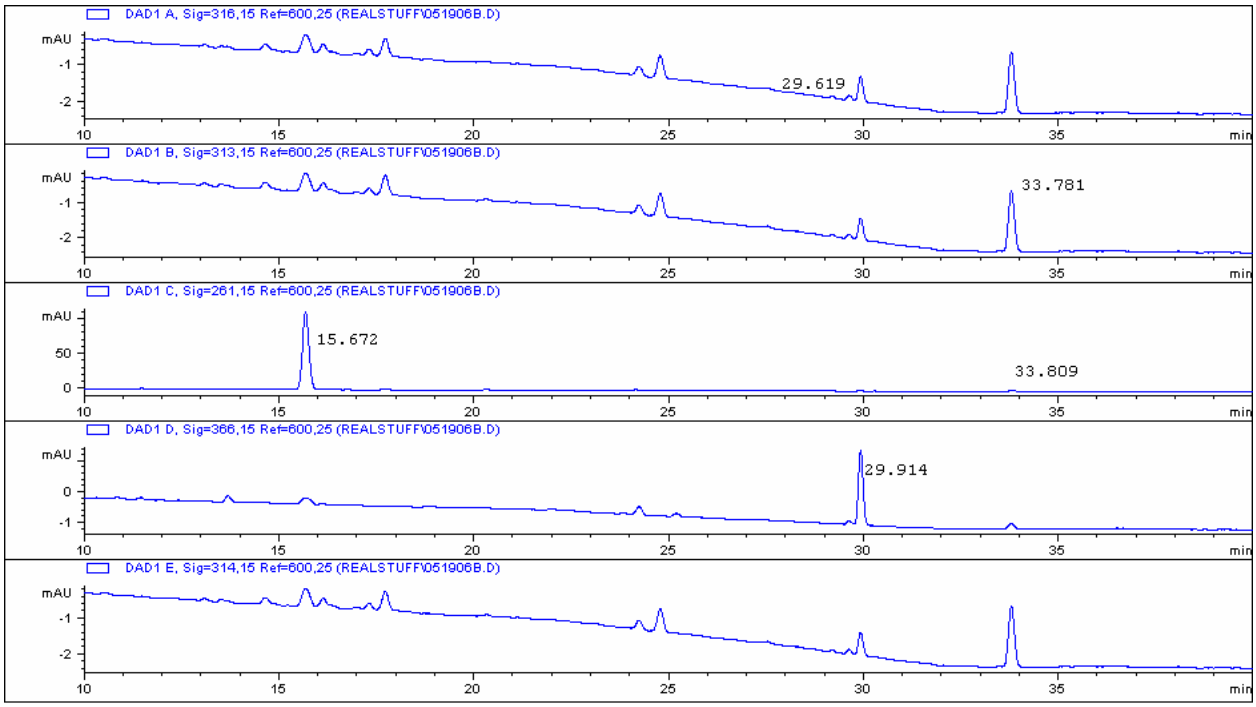


Figure 4.26 HPLC chromatogram of 7 day culture containing Disperse Red 13 — veratryl alcohol (15.672 min, 261 nm), 1,2-dimethoxy-4-nitrobenzene (29.691 min, 316 nm), 2-chloro-4-nitrooaniline (29.914 min, 366 nm), 2-chloro-4-nitroanisole (33.781 min, 313 nm), 1-chloro-3-nitrobenzene (33.809 min, 261 nm).

4.2 The influence of initial dye concentration on degradation of Disperse Red 1 by *Pleurotus ostreatus*

In order to test how the initial dye concentration of Disperse Red 1 can influence the degradation results, three initial dye concentrations, 50 ppm, 100 ppm and 200 ppm were tested. The amounts of three major products, nitrobenzene, 4-nitroaniline and 4-nitrophenol along with 4-nitroanisole were quantitatively monitored by HPLC.

The dye concentration did not significantly affect the production of nitrobenzene from Disperse Red 1 (Figure 4.27). Cultures having different initial dye concentrations all showed a constant increase of nitrobenzene, and there was no obvious difference between the cultures in

terms of nitrobenzene concentration. The constant increase of nitrobenzene indicated nitrobenzene may not be further degraded by the fungus.

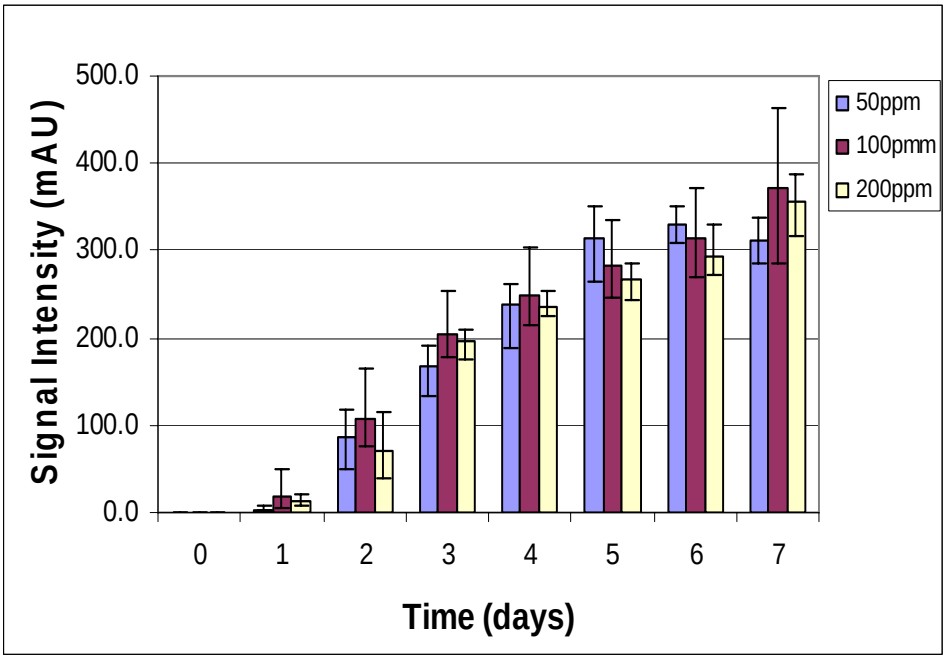


Figure 4.27 Production of Nitrobenzene from Disperse Red 1 with different initial dye concentrations

For 4-nitrophenol, higher initial dye concentrations showed higher levels of 4-nitrophenol (Figure 4.28). For 50 ppm of dye, an obvious decrease was seen after 4 days degradation which suggested that 4-nitrophenol may be further degraded into other compounds. Such a decrease was observed after 5 days for 100 ppm and after 7 days for 200 ppm. It was reasonable that cultures having 100 ppm and 200 ppm initial dye concentration exhibited the decrease of 4-nitrophenol later than for 50 ppm because 100 ppm and 200 ppm had more 4-nitrophenol

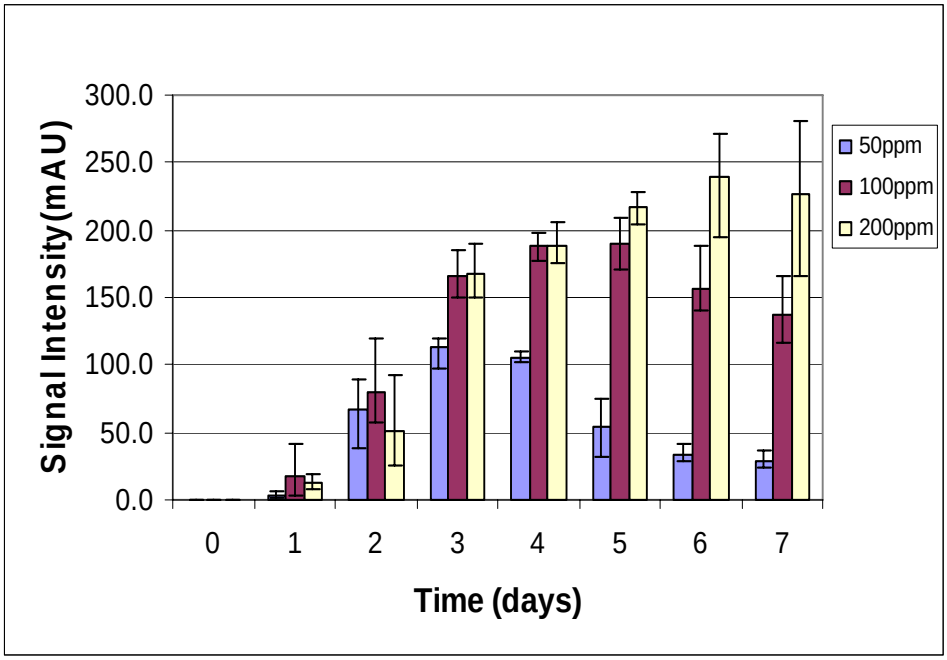


Figure 4.28 Production of 4-nitrophenol from Disperse Red 1
with different initial dye concentrations

produced and it took a longer time for the culture to transform 4-nitrophenol into other compounds. Four-nitrophenol has been widely used in manufacture of pharmaceuticals, dyes, pesticides etc. It is probably the most important compound among the mono-nitrophenols that cause serious environmental contamination (Donlon et al., 1996, Karin and Gupta, 2002). As for biodegradation, most of the work has been done by using 4-nitrophenol as a sole carbon and nitrogen source by various bacteria (Jain et al., 1994; Kadiyala et al., 1998, Bhushan et al., 2000; Chauhan et al., 2000, Roldán et al., 1998). The reduction of 4-nitrophenol by *Pleurotus ostreatus* is herein reported for the first time. This suggests that the white rot fungus may also be used in biodegradation to solve the environmental concerns brought about by 4-nitrophenol.

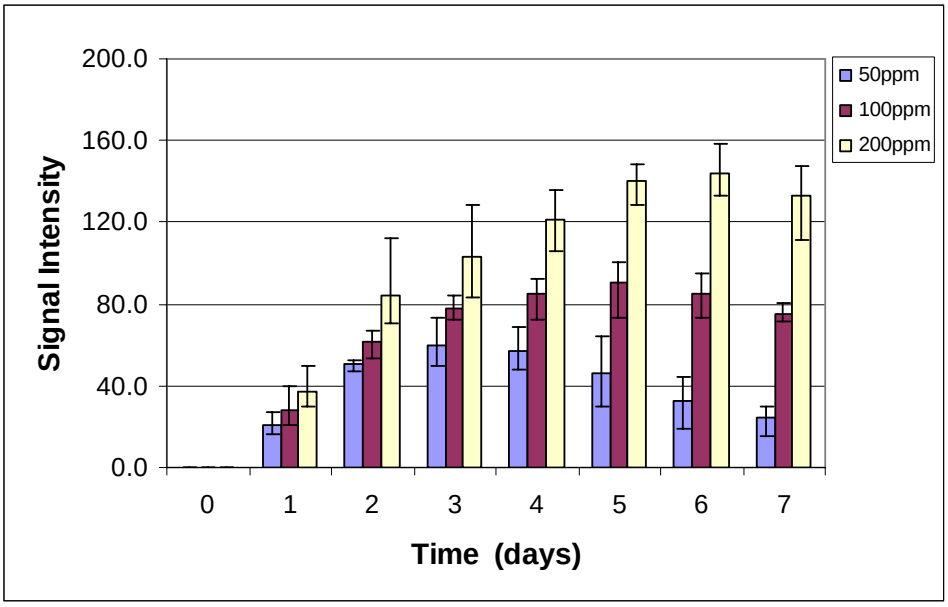


Figure 4.29 Production of 4-nitroaniline from Disperse Red 1
with different initial dye concentrations

Figure 4.29 illustrates that the production of 4-nitroaniline is also influenced by the initial dye concentration. Fifty ppm starting dye concentration showed a decrease of 4-nitroaniline after 4 days degradation, while such a decrease occurred after 6 and 7 days, respectively, for 100 ppm and 200 ppm starting dye concentrations. As in the case of 4-nitrophenol, more 4-nitroaniline was produced in the cultures initially having 100 ppm and 200 ppm dye than in ones having 50 ppm dye, therefore much more time was taken for the degradation of 4-nitroaniline into descendant compounds to overwhelm the production of 4-nitroaniline and cause its amount to decrease. Four-nitroaniline has also been considered as a serious environmental contamination, and it has been detected in a wastewater of the production of azo dyes (Sarasa et al., 1998). However, there are only a few reports of successful elimination or degradation of 4-nitroaniline in aerobic wastewater treatment processes (Saupe 1999). Instead, physicochemical treatments such as ozonization and the photo-Fenton process have been typical means of degradation of 4-nitroaniline (Wang and Jin, 1988; Ruppert and Baucer 1993). Saupe (1999)

developed a continuous bioreactor system, in which bacteria collected from a waste water bio-sludge was fed with 4-nitroaniline as the sole carbon and nitrogen, that achieved a high efficiency of degradation of 4-nitroaniline. To date no research has been done using the white rot fungus to degrade 4-nitroaniline. The reduction of 4-nitroaniline was observed for all cultures having different initial dye concentrations, which indicated that *Pleurotus ostreatus* may also be employed to achieve a biodegradation of 4-nitroaniline.

Regarded as a secondary product from 4-nitrophenol, 4-nitroanisole may also be produced directly from the unstable intermediate nitrophenyldiazene. Cultures with 100 ppm and 200 ppm illustrated (Figure 4.30) constant increases of 4-nitroanisole after 2 days, while 50 ppm showed a decrease in 4-nitroanisole after 5 days. This suggested that 4-nitroanisole can be degraded by *Pleurotus ostreatus*.

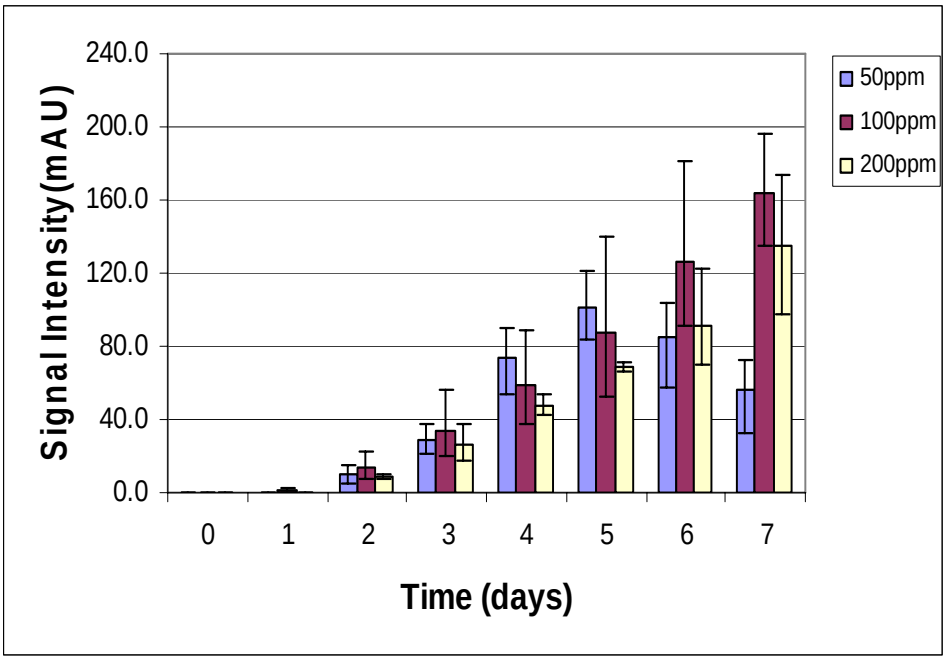


Figure 4.30 Production of 4-nitroanisole from Disperse Red 1 with different initial dye concentrations

4.3 Secondary degradation of the primary products from the hydrophobic azo dyes.

Since we have found that two of the three primary products from Disperse Red 1 could be degraded further, experiments were carried out to determine the nature of the degradations.

4.3.1 Secondary degradation of the primary product nitrobenzene

The chromatograms of 1 and 4 day cultures containing only nitrobenzene showed the existence of nitrobenzene and veratryl alcohol (Figure 4.31 and 4.32). Although the 7 day culture gave some additional small peaks at time ranging from 17 to 22 min, their structure information was not determined (Figure 4.33).

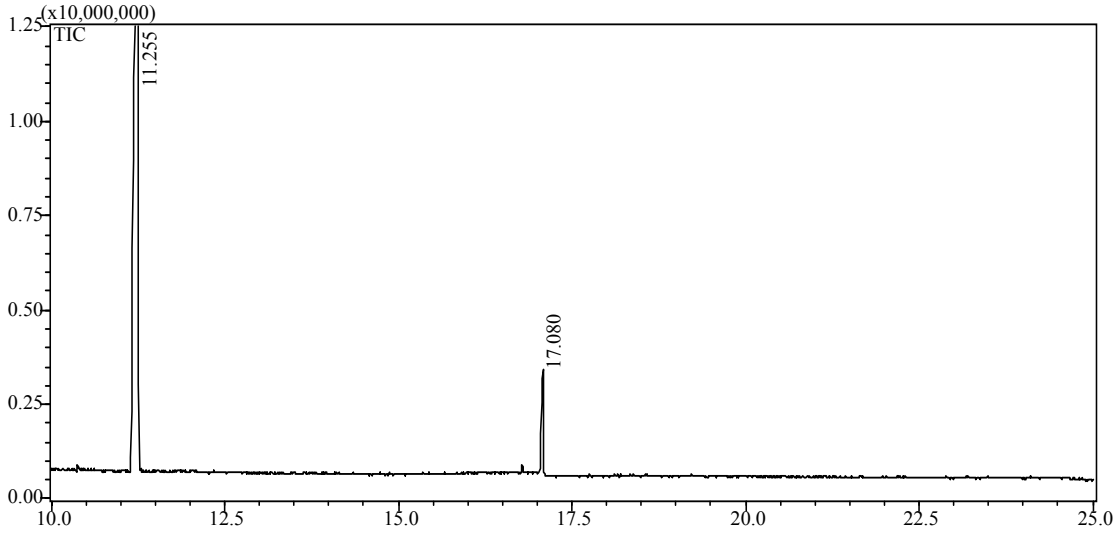


Figure 4.31 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 1 day culture containing nitrobenzene — nitrobenzene (11.255min), veratryl alcohol (17.080 min).

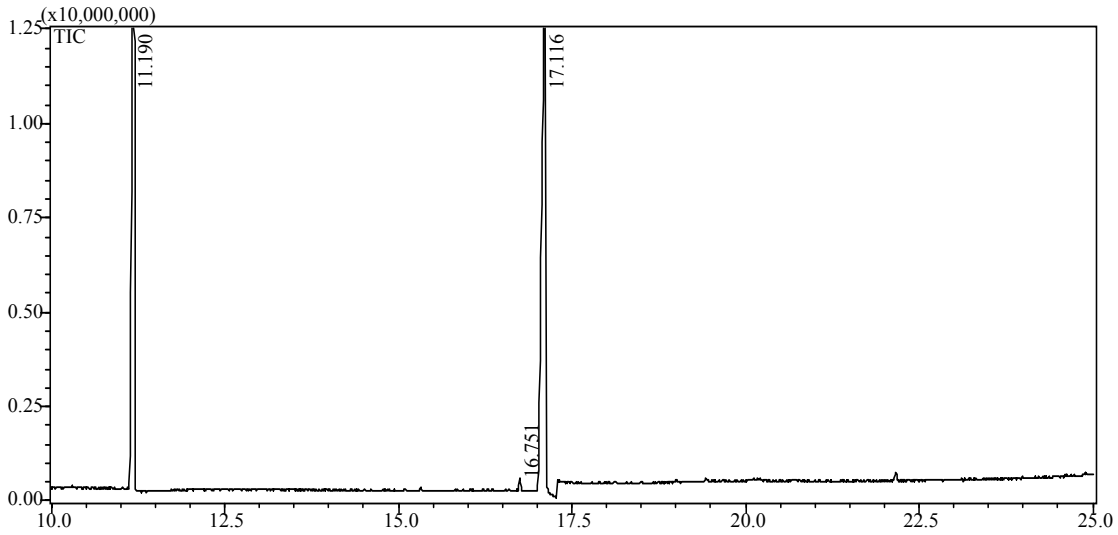


Figure 4.32 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 4 day culture containing nitrobenzene — nitrobenzene (11.190min), veratryl aldehyde (16.751 min), veratryl alcohol(17.116 min).

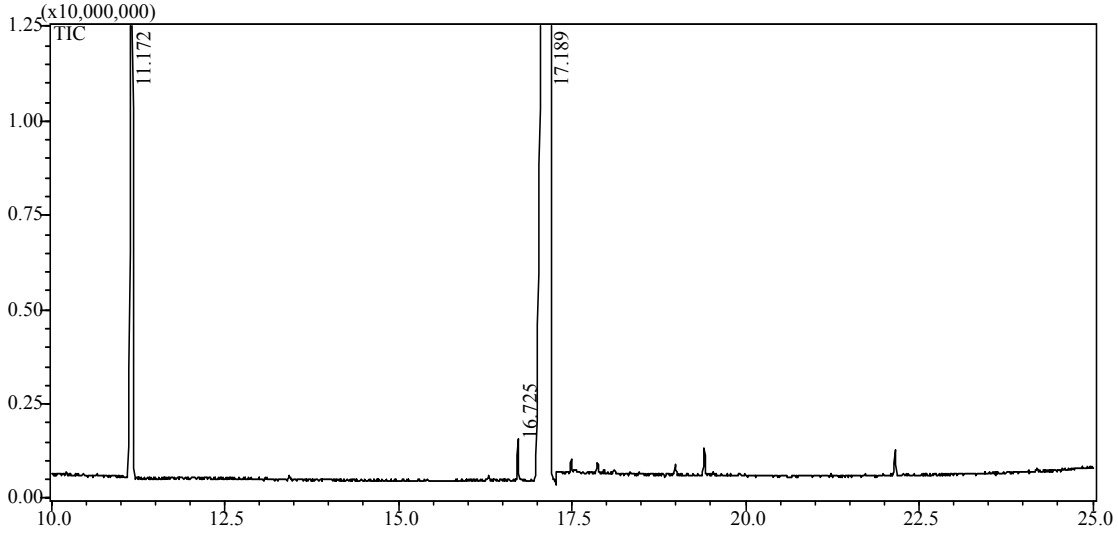


Figure 4.33 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 7 day culture containing nitrobenzene — nitrobenzene (11.171min), veratryl aldehyde (16.725 min), veratryl alcohol (17.189min).

4.3.1 Secondary degradation of the primary product 4-nitrophenol and the influence of initial concentration

The chromatogram of the 1 day 4-nitrophenol degradation culture clearly showed that 4-nitroanisole was produced from 4-nitrophenol (Figure 4.34). After 4 days degradation, two more compounds, 2,4-dinitrophenol (16.967min) and 2,4-nitroanisole (20.119min), were detected (Figure 4.35). The source of the nitro group added onto the benzene ring is still not clear. However, in the research done using bacteria *Rhodococcus wratislaviensis* for the degradation and detoxification of 4-nitrophenol, nitrite was released during the degradation (Gemini et al, 2005). Likewise, nitrite may also be released during the degradation of 4-nitrophenol by *Pleurotus ostreatus*. The nitrite can possibly be the source of the nitro group added onto the benzene ring. The 2,4-dinitroanisole was considered to be transformed from 2,4-dinitrophenol by a methylation step similar to that of 4-nitrophenol to 4-nitroanisole. This hypothesis was later tested in a separate experiment.

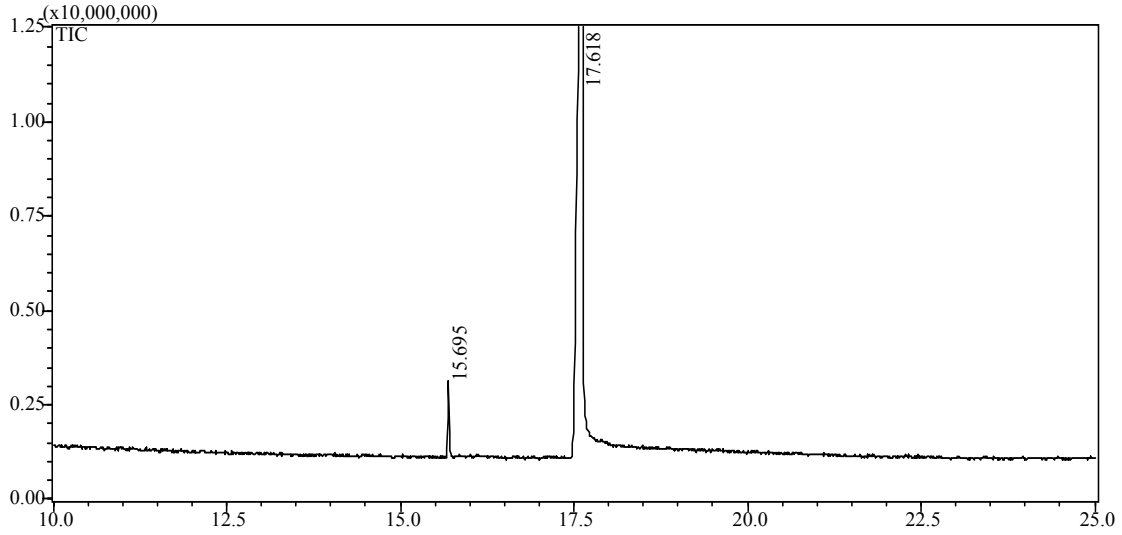


Figure 4.34 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 1 day culture containing 4-nitrophenol — 4-nitroanisole (15.695min), 4-nitrophenol (17.618 min).

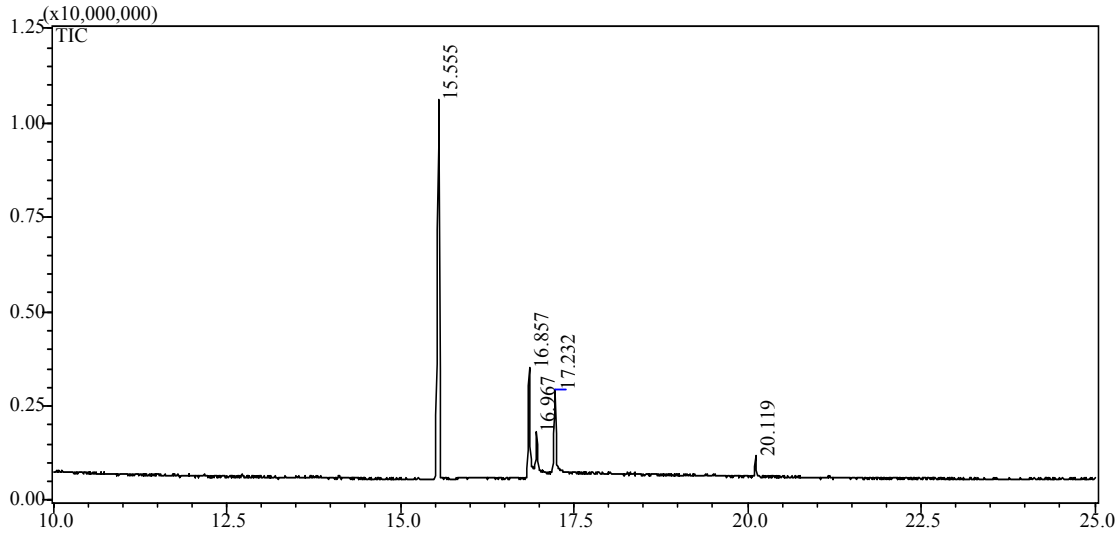


Figure 4.35 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 4 day culture containing 4-nitrophenol — 4-nitroanisole (15.555 min), veratryl alcohol (16.857 min), 2,4-dinitrophenol (16.967 min), 4-nitrophenol (17.232 min), 2,4-dinitroanisole (20.119 min).

The gas chromatograms of the 7 day culture, both TIC and SIC displays (Figure 4.36 and 4.37), along with the HPLC results (Figure 4.38) of 7 day culture, confirmed that 4-nitroanisole, 2,4-dinitrophenol and 2,4-dinitroanisole were produced from 4-nitrophenol. Another compound, 1,2-dimethoxy-4-nitrobenzene (18.280min), was detected. This compound was previous detected in cultures containing only dyes, including Disperse Red 1, Disperse Red 19, Disperse Orange 25 and Disperse Red 13. It is suggested that the transformation of 1,2-dimethoxy-4-nitrobenzene from 4-nitrophenol was responsible for the detection of 1,2-dimethoxy-4-nitrobenzene in dye degradation cultures. However, although the degradation of Disperse Red 13, does not produce 4-nitrophenol, 1,2-dimethoxy-4-nitrobenzene was nevertheless detected in the 7 day sample. This suggests that 1,2-dimethoxy-4-nitrobenzene may be produced by another pathway not involving 4-nitrophneol.

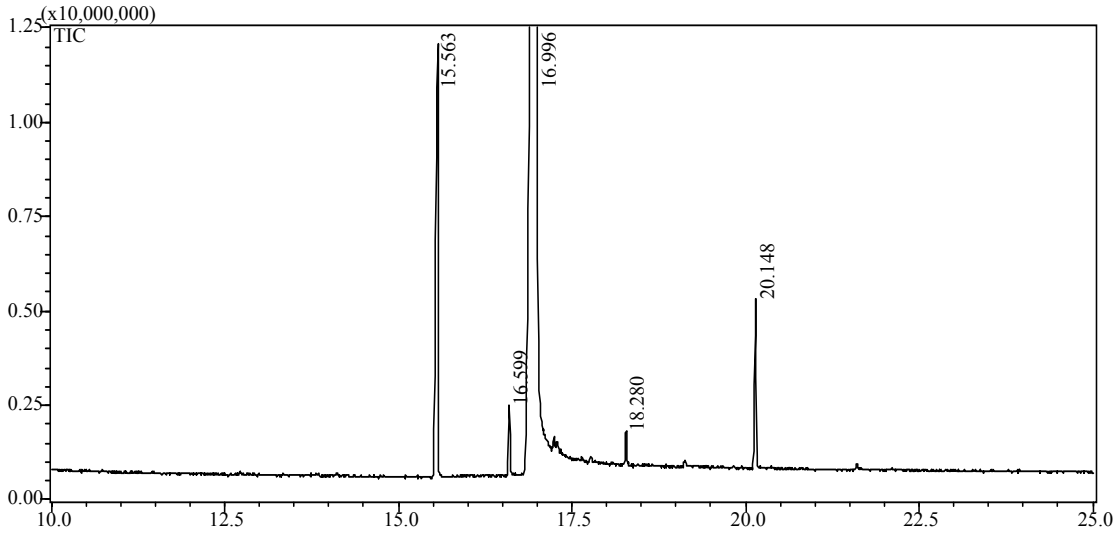


Figure 4.36 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 7 day culture containing 4-nitrophenol — 4-nitroanisole (15.563 min), veratryl aldehyde (16.599 min), veratryl alcohol (16.996 min), 1,2-dimethoxy-4-nitrobenzene (18.280 min), 2,4-dinitroanisole (20.148 min).

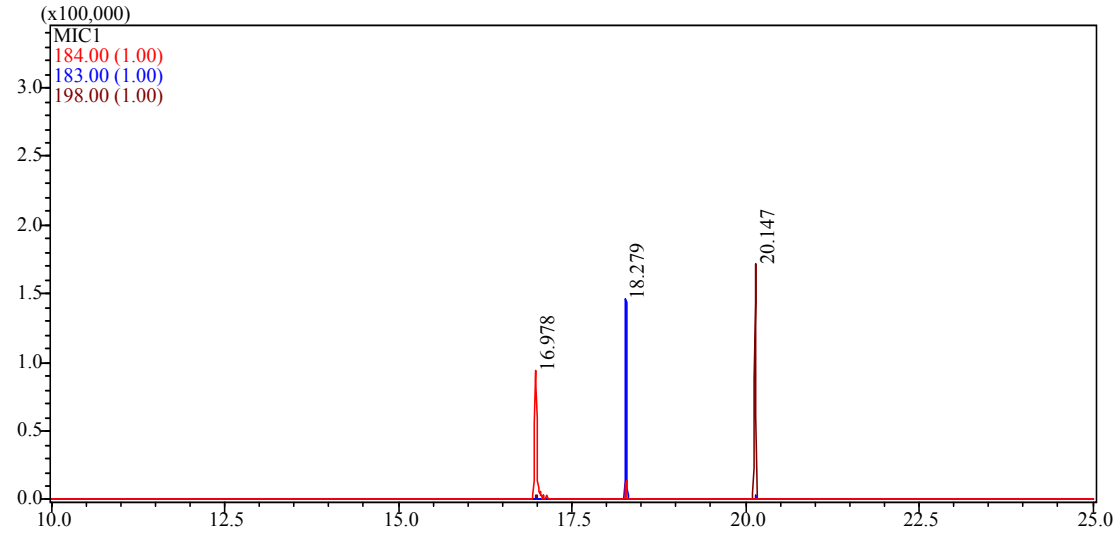


Figure 4.37 X axis (retention time), Y axis (signal intensity). SIC display (m/z 183, 184 and 198) of gas chromatogram of 7 day old degradation culture of 4-nitrophenol — 2,4-dinitrophenol (16.978min, m/z 184), 1,2-dimethoxy-4-nitrobenzene (18.279min, m/z183), 2,4-dinitroanisole (20.147min, m/z 198)

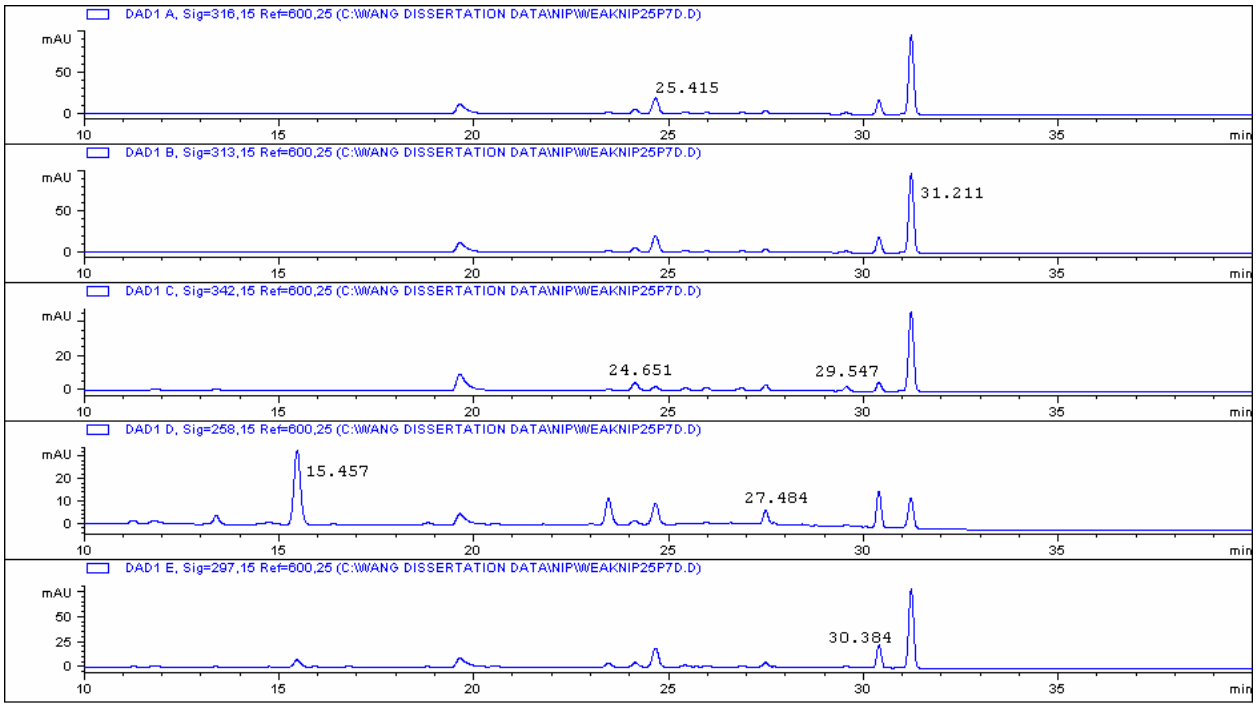


Figure 4.38 HPLC chromatogram of 7 day degradation culture of 4-nitrophenol — veratryl alcohol (15.457 min, 258 nm), veratryl aldehyde (24.651 min, 342 nm), 4-nitrophenol (25.414 min, 316 nm), 2,4-dinitrophenol (27.484 min, 258 nm),1,2-dimethoxy-4-nitrobenzene (29.547 min, 342 nm), 2,4-dinitroanisole (30.384 min, 297 nm), 4-nitroanisole (31.211, 316 nm).

It has been shown that the initial dye concentration can affect the degradation results. This brought up the question as to the initial concentration of 4-nitrophenol, when taken as starting compound, may also affect the degradation results. To test this, pure 4-nitrophenol powder was added to the cultures to give initial concentrations at 25ppm, 50ppm and 100ppm. The amount of 4-nitrophenol and its degradation products were monitored by HPLC every day for 7 days.

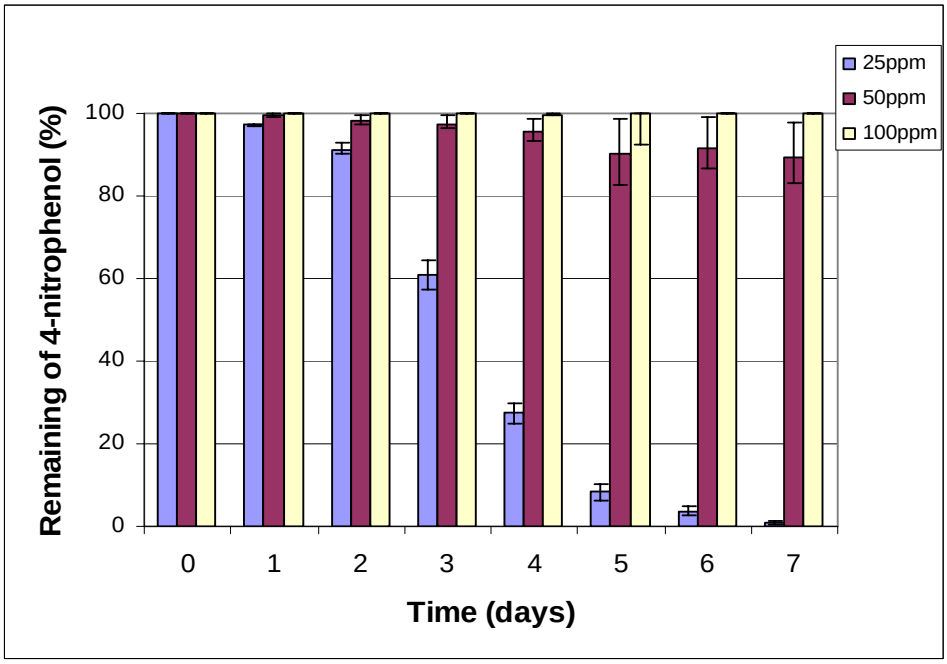


Figure 4.39 Reduction of 4-nitrophenol with different initial concentrations by *Pleurotus ostreatus*

The results show that degradation did not occur at 100 ppm 4-nitrophenol. The amount of 4-nitrophenol did not change after 7 days exposure (Figure 4.39). Cultures initially having 50 ppm 4-nitrophenol removed about 10 percent of the total 4-nitrophenol and gave fewer degradation products than 25 ppm ones. Cultures having 25 ppm initial 4-nitrophenol illustrated very effective reduction of 4-nitrophenol. After 5 days degradation, almost 90 percent of the 4-nitrophenol was degraded. Less than one percent still remained in the culture after 7 days degradation. These results show that an initial 4-nitrophenol concentration as high as 100 ppm may be too toxic for the fungus to undergo any degradation. The white rot fungus *Pleurotus ostreatus* may be a potential degrader for the environmental 4-nitrophenol, but only if a low concentration exists. In addition, more research is needed to study the products from the 4-nitrophenol degradation by *Pleurotus ostreatus,* and their toxicities.

Figure 4.40 shows the transformation of 4-nitrophenol to 4-nitroanisole after introduction of the former to fungal culture. Only the cultures having starting concentration of 25 ppm and 50 ppm were able to produce 4-nitroanisole. When the initial 4-nitrophenol was raised to 100ppm, no 4-nitroanisole was detected. In the cultures initially having 25 ppm of 4-nitrophenol, the amount of 4-nitroanisole constantly increased until 6 days, when 4-nitrophenol was almost totally removed. The 50 ppm samples also exhibited a constant increase of 4-nitroanisole in the culture, but with much variation.

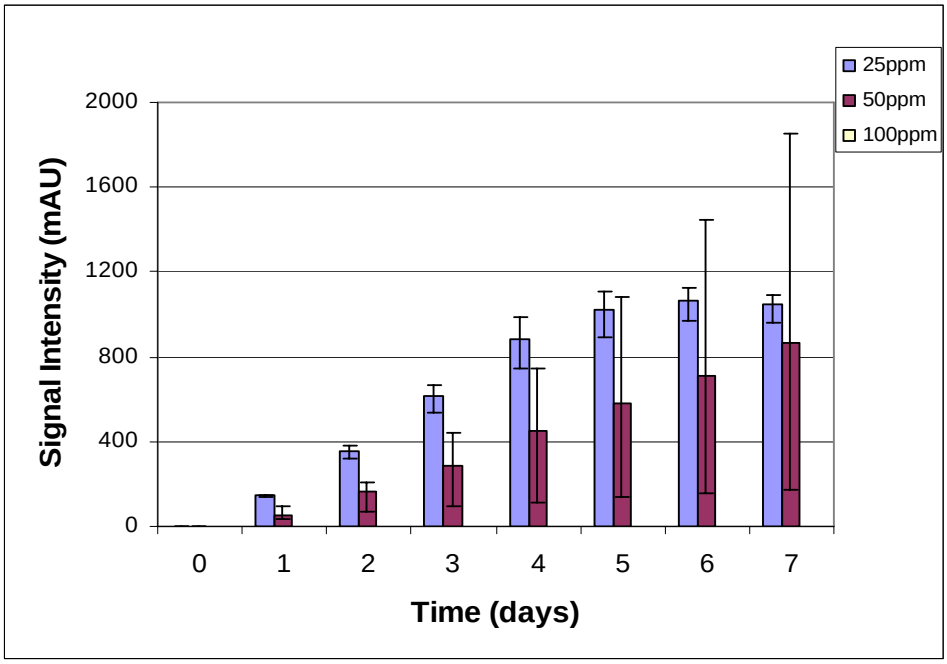


Figure 4.40 Production of 4-nitroanisole from 4-nitrophenol with different initial concentrations

Standard 4-nitrophenol introduced to the fungal culture produced 1,2-dimethoxy-4-nitrobenzene (Figure 4.41). Cultures initially having 4-nitrophenol at 25 ppm and 50 ppm both showed production of 1,2-dimethoxy-4-nitrobenzene, while the 100 ppm samples did not produce any 1,2-dimethoxy-4-nitrobenzene.

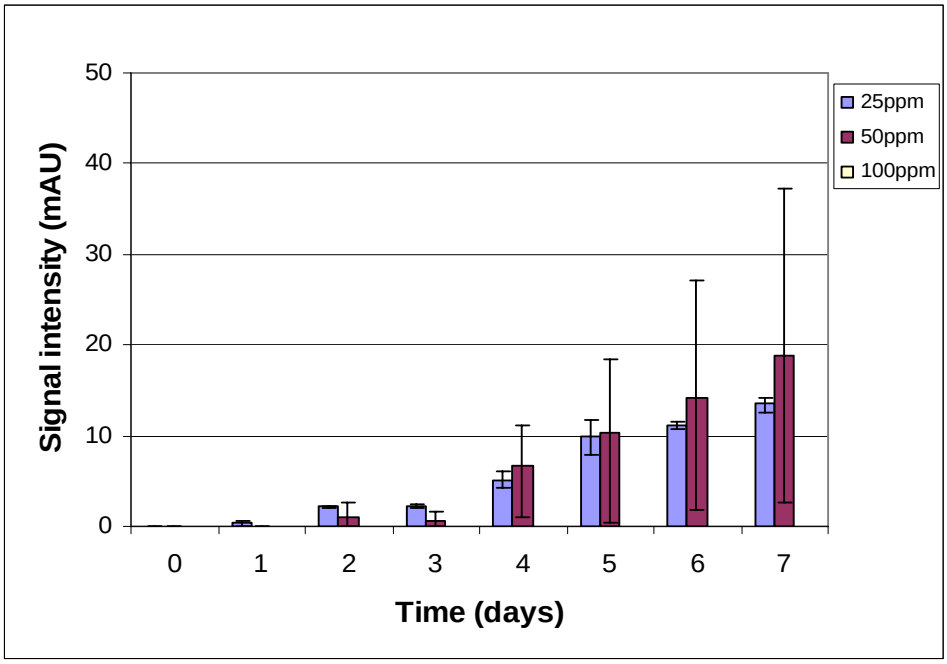


Figure 4.41 Production of 1,2-dimethoxy-4-nitrobenzene from 4-nitrophenol with different initial concentrations

In Figure 4.42 and 4.43, 2,4-dinitrophenol and 2,4-dinitroanisole were only detected in the cultures the 25 ppm of 4-nitrophenol. The decrease of 2,4-dinitrophenol after 5 days indicated it was degraded to another compound, quite possibly 2,4-dinitroanisole, through a methylation step as mentioned earlier.

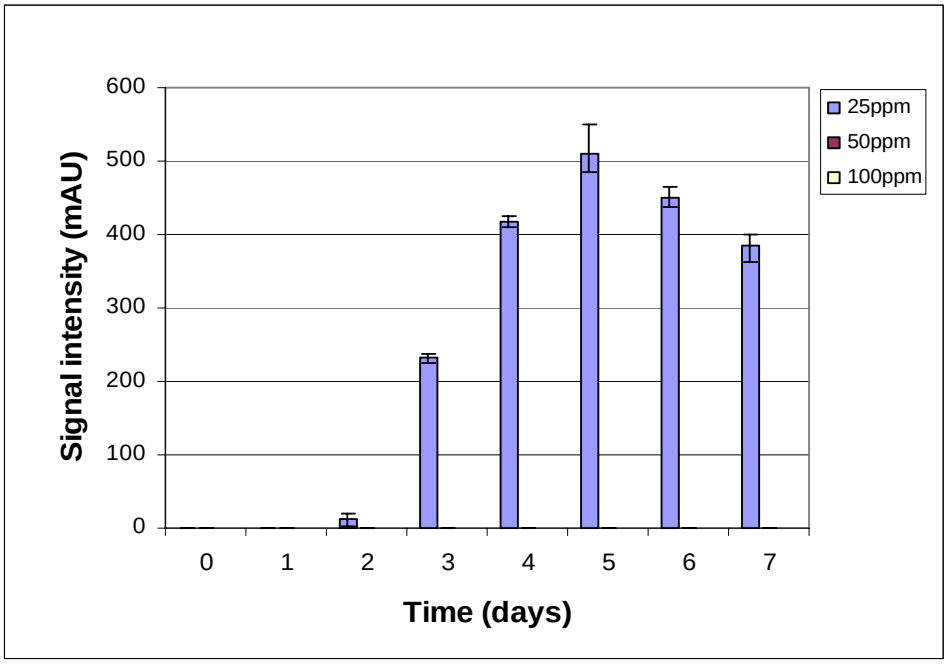


Figure 4.42 Production of 2,4-dinitrophenol from 4-nitrophenol with different initial concentrations

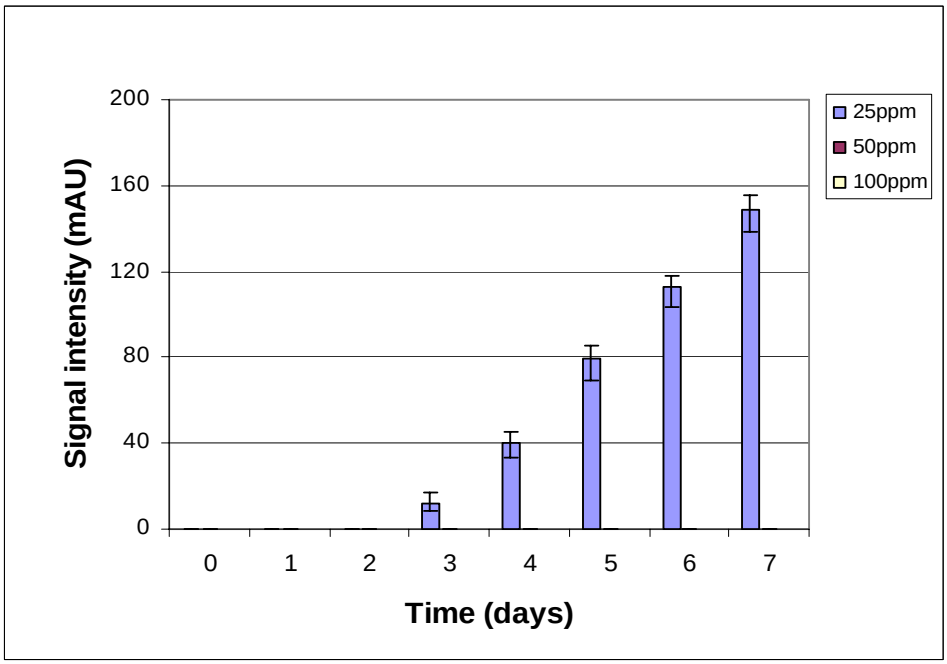


Figure 4.43 Production of 2,4-dinitroanisole from 4-nitrophenol with different initial concentrations

Given the results discussed, it was concluded that the initial concentration of 4-nitrophenol played a very important role in the degradation. A relatively high concentration may lead to lower fungal growth, lower level of enzyme production and therefore less degradation effectiveness. In cultures having an initial concentration of 50 ppm 4-nitrophenol, another compound 4-nitroguaiacol (2-methoxy-4-nitrophenol) was detected (Figure 4.44), but was not detected in either the 25 ppm or 100 ppm samples. The reaction that was responsible for production of 4-nitroguaiacol apparently was a reaction competitive with the production of 2,4-dinitrophenol since they were attacking the same position on the benzene ring in 4-nitrophenol. Nevertheless, the production of 4-nitroguaiacol and 2,4-nitrophenol suggested that the meta-position to the hydroxyl group on the benzene ring in 4-nitrophenol is a very active site that can accept some small functional groups and form new compounds.

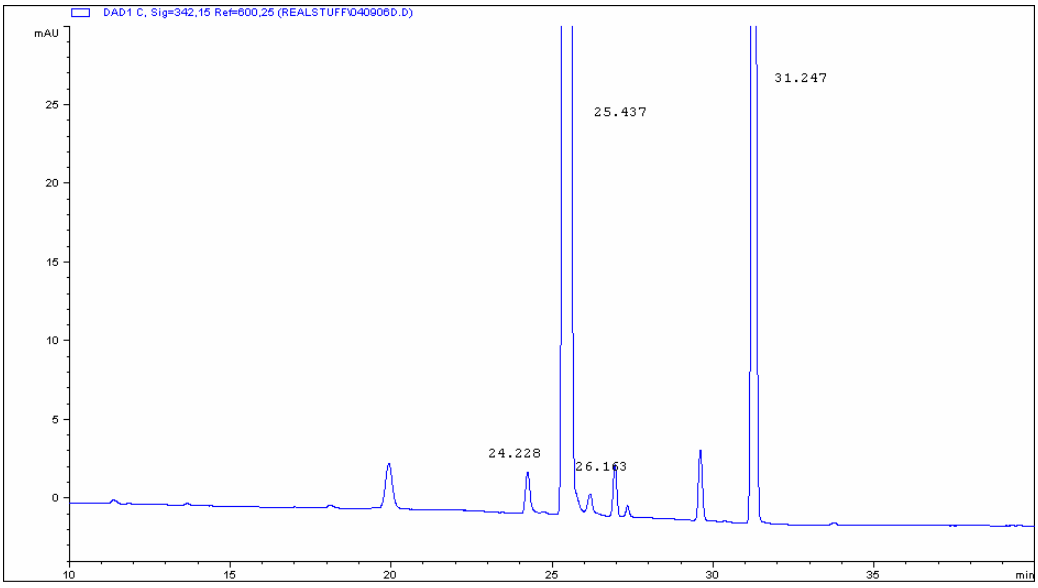


Figure 4.44 HPLC chromatogram of 7 day culture initially containing 50ppm 4-nitrophenol (342 nm), veratryl aldehyde (24.228 min), 4-nitrophenol (25.437 min), 4-nitroguaiacol (26,613 min), 4-nitroanisole (31.247 min)

4.3.3 Secondary degradation of the primary product 4-nitroaniline and the influence of initial concentration

During the fungal degradation of Disperse Red 1, 4-nitroaniline was found to decrease after several days. It was believed that further degradation had occurred to the 4-nitroaniline in the fungal culture. Thus pure 4-nitroaniline was added to a *Pleurotus ostreatus* culture as the starting compound at 25 ppm initial concentration. The possible degradation compounds were identified by GC/MS and HPLC.

The gas chromatogram of 1 day culture gave only two peaks that were assigned to veratryl alcohol and the starting compound 4-nitroaniline (Figure 4.45). However, after 4 days degradation, 3 products were identified. They were nitrobenzene, 4-nitroanisole and 4-nitrophenol (Figure 4.46). An unknown metabolite at about 21.162 min was also detected, but the structure was not clarified.

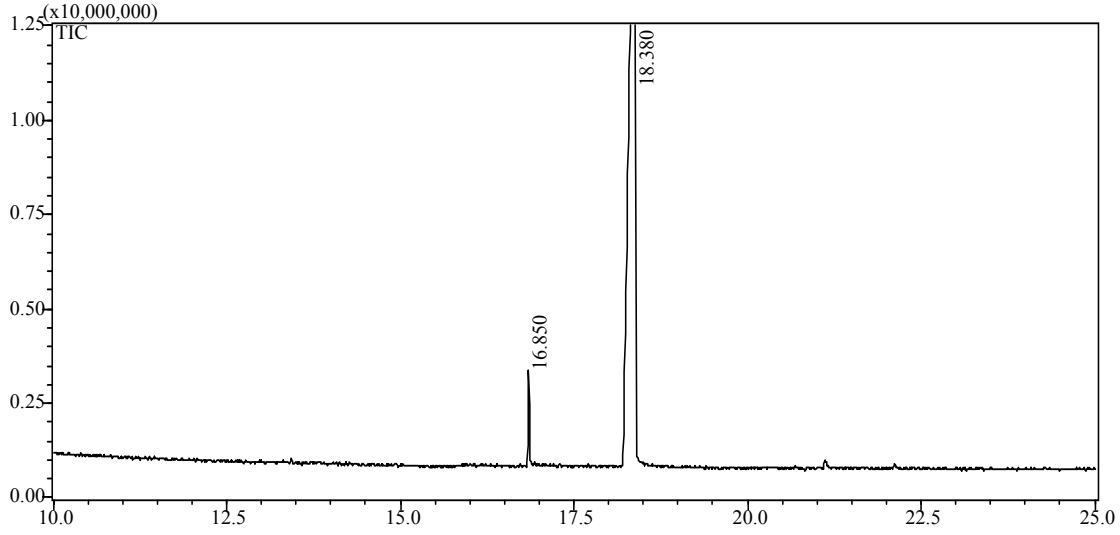


Figure 4.45 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 1 day culture having 4-nitroaniline — veratryl alcohol (16.850min), 4-nitroaniline (18.380min)

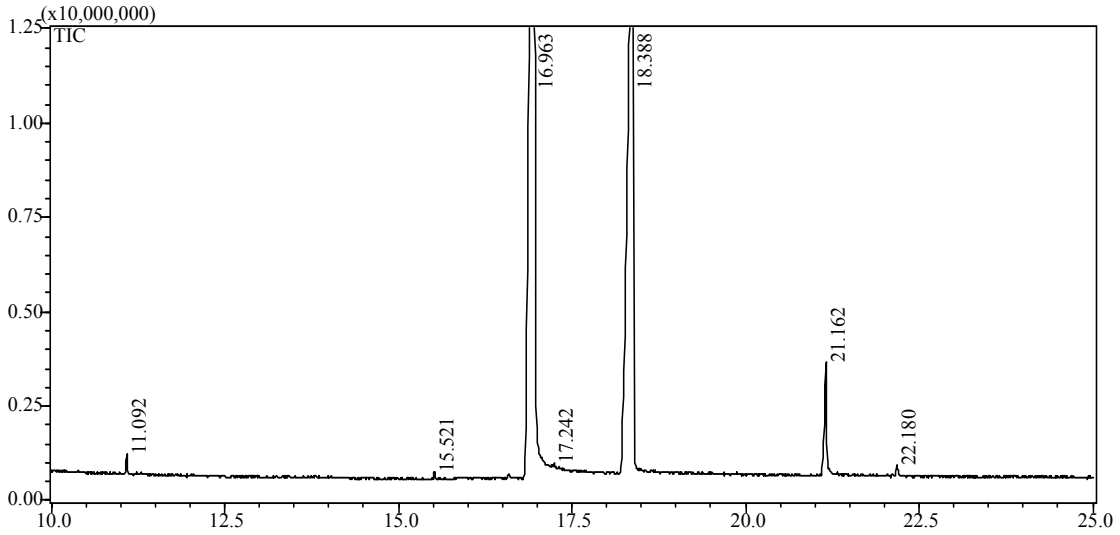


Figure 4.46 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 4 day culture containing 4-nitroaniline — nitrobenzene (11.092min), 4-nitroanisole (15.521min), veratryl alcohol (16.963min), 4-nitrophenol (17.242min), 4-nitroaniline(18.388min), unknown metabolite (21.162min)

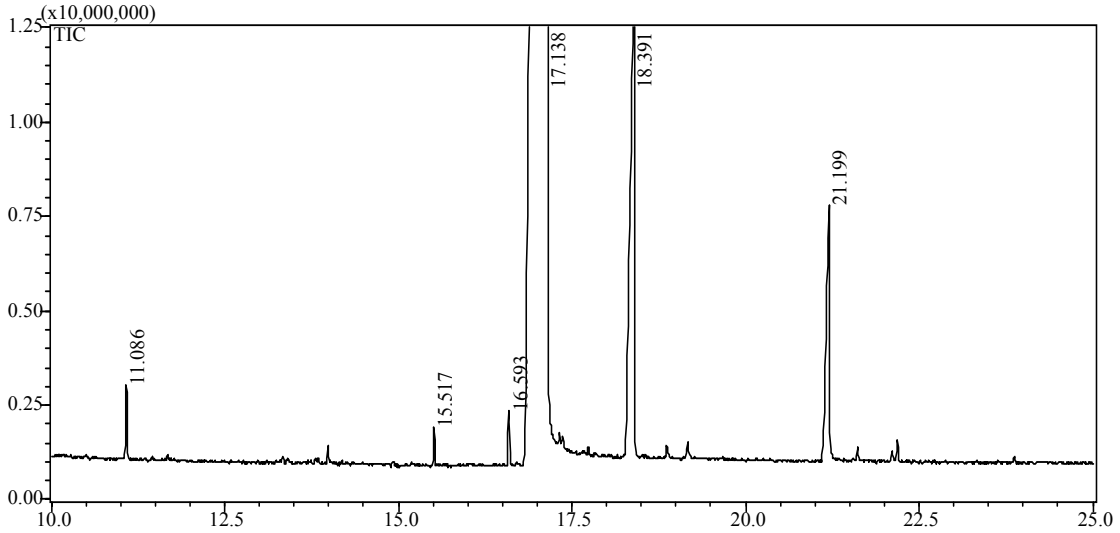


Figure 4.47 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 7 day culture having 4-nitroaniline ― nitrobenzene (11.086 min), 4-nitroanisole (15.517 min), veratryl aldehyde (16.593 min), veratryl alcohol (17.138 min), 4-nitroaniline(18.391 min), unknown metabolite (21.199min)

The gas chromatogram of the 7 day culture confirmed the production of nitrobenzene and 4-nitroanisole with several more unknown peaks (Figure 4.47). The HPLC chromatogram was in accordance with the results given by GC/MS and confirmed that 4-nitrophenol was in the 7 day degradation culture (Figure 4.48). A compound that was detected earlier during 4-nitrophenol degradation, 1,2-dimethoxy-4-nitrobenzene, was again detected in 4-nitroaniline degradation cultures. It is still not clear whether the production of 4-nitroanisole and 1,2-dimethoxy-4-nitrobenze were due to the 4-nitrophenol, which was also produced in 4-nitroaniline culture, or if there is another mechanism responsible for their formation. However, the formation of nitrobenzene and 4-nitrophenol indicated that 4-nitroaniline might lose an ammonia molecule to form a free radical that later accepted a hydrogen or hydroxyl radical to

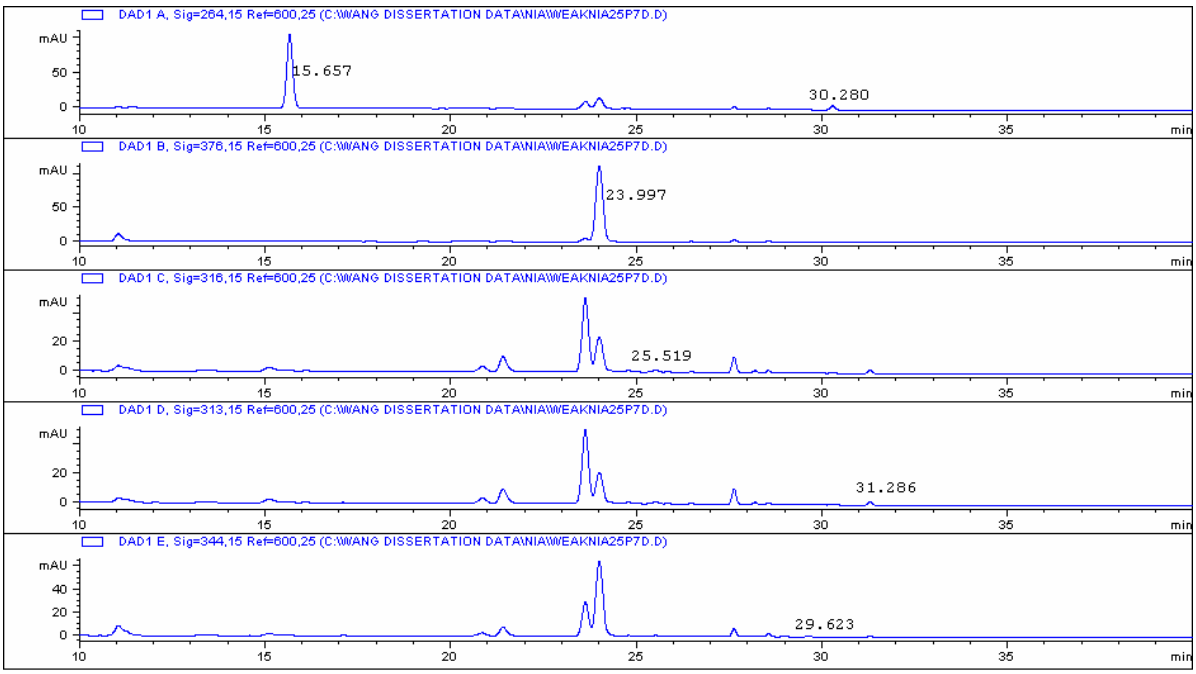


Figure 4.48 HPLC chromatogram of 7 day culture having 4-nitroaniline — veratryl alcohol (15.657 min, 264 nm), 4-nitroaniline(23.997 min, 376 nm), 4-nitrophenol (25.519 min, 316nm), 1,2-dimethoxy-4-nitrobenzene (29.623 min, 344 nm), nitrobenzene (30,280 min, 264nm), 4-nitroanisole (31.286 min, 313 nm).

form nitrobenzene or 4-nitrophenol (Figure 4.49). Nevertheless, 4-nitrophenol did not follow a similar way to form nitrobenzene, since no nitrobenzene was detected in the 4-nitrophenol degradation culture.

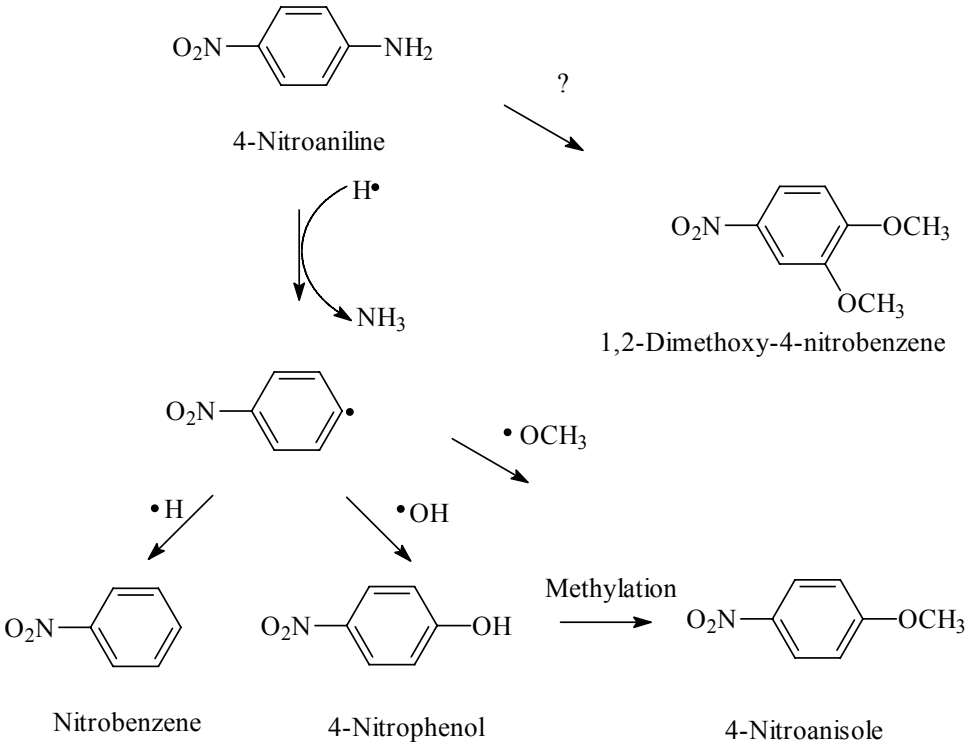


Figure 4.49 A schematic pathway for degradation of 4-nitroaniline by *Pleurotus ostreatus*

As the initial concentration of 4-nitrophenol was found to greatly influence the degradation, it was reasonable to examine whether the initial concentration of 4-nitroaniline affect the degradation as well. Pure 4-nitroaniline was added to culture aliquots to give three different initial concentrations at 25 ppm, 50 ppm and 100 ppm. The amount of 4-nitroaniline and its degradation products was monitored by HPLC for 7 days. The cultures having 25 ppm 4-nitroaniline showed the most degradation efficiency with more than 40 percent of 4-nitroaniline degraded after 7 days. About 40 percent of the total 4-nitroaniline was degraded in the culture

with initial concentration of 50 ppm, while only 20 percent of 4-nitroaniline was degraded in the 100ppm sample. Unlike the case of 4-nitrophenol, for which 100 ppm starting concentration completely depressed the degradation, a starting concentration at 100 ppm of 4-nitroaniline still showed some degradation despite being a relatively low percent.

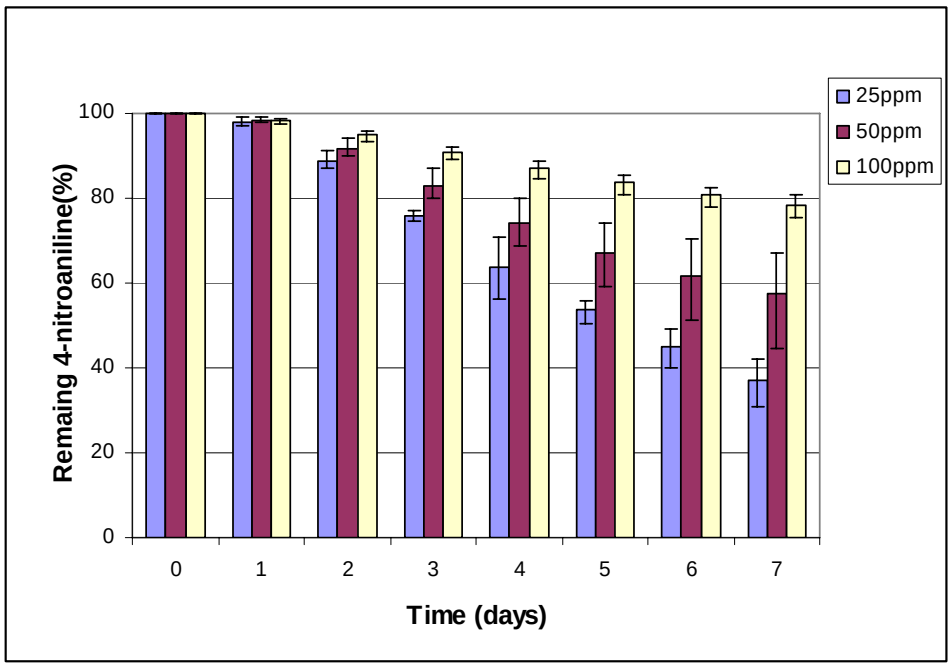


Figure 4.50 Reduction of 4-nitroaniline with different initial concentrations

No clear influence on production of 4-nitrobenzene was found from the different initial concentrations (Figure 4.51). The cultures all gave a constant increase of nitrobenzene with time, with the 100 ppm sample lagging behind the other two conditions. The constant increase of nitrobenzene in the cultures indicated that nitrobenzene is not further degraded by the fungus.

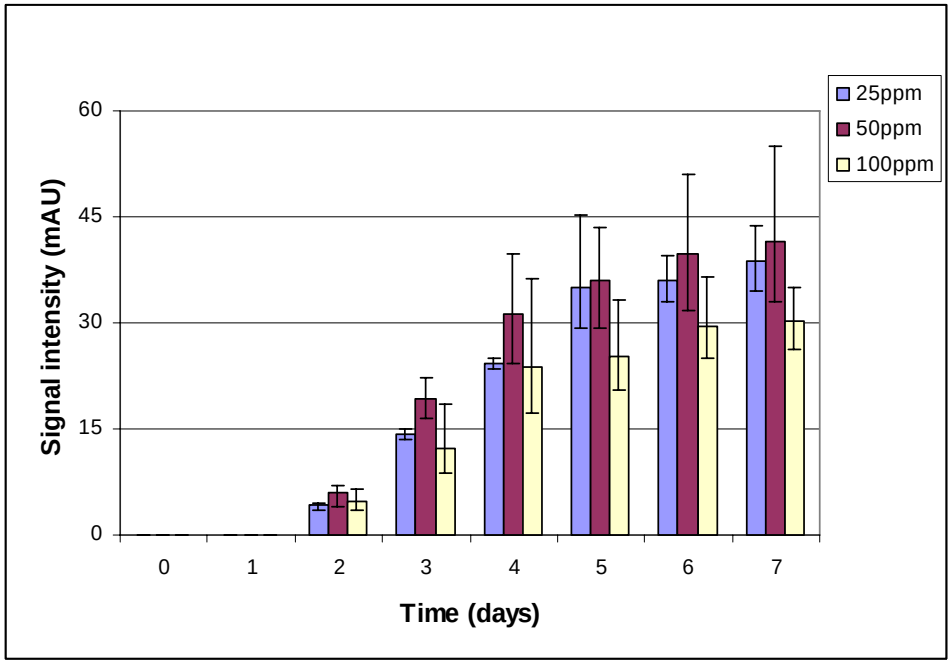


Figure 4.51 Production of 4-nitrobenzene from 4-nitroaniline with different initial concentrations

As was mentioned, the production of 4-nitroanisole in cultures initially only having 4-nitroaniline is quite complex, since 4-nitroanisole can be produced from 4-nitrophenol, which itself was also produced from 4-nitroaniline. In addition, 4-nitroaniline may transform into 4-nitroanisole without going through the formation of 4-nitrophenol. A higher starting concentration of 4-nitroaniline did produce a higher level of 4-nitrophenol, but the amount of 4-nitrophenol in all cultures reached a maximum level after four or five days, and then decreased to certain levels. Such a trend can easily be explained by the fact that 4-nitrophenol can be readily further degraded by *Pleurotus ostreatus*, especially at a low concentration (Figure 4.52).

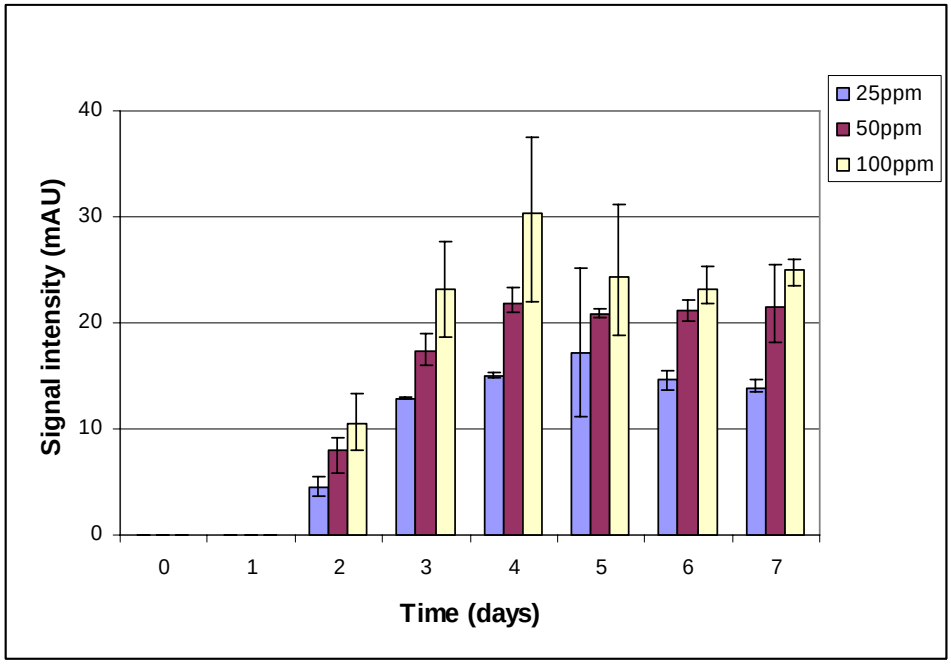


Figure 4.52 Production of 4-nitrophenol from 4-nitroaniline with different initial concentrations

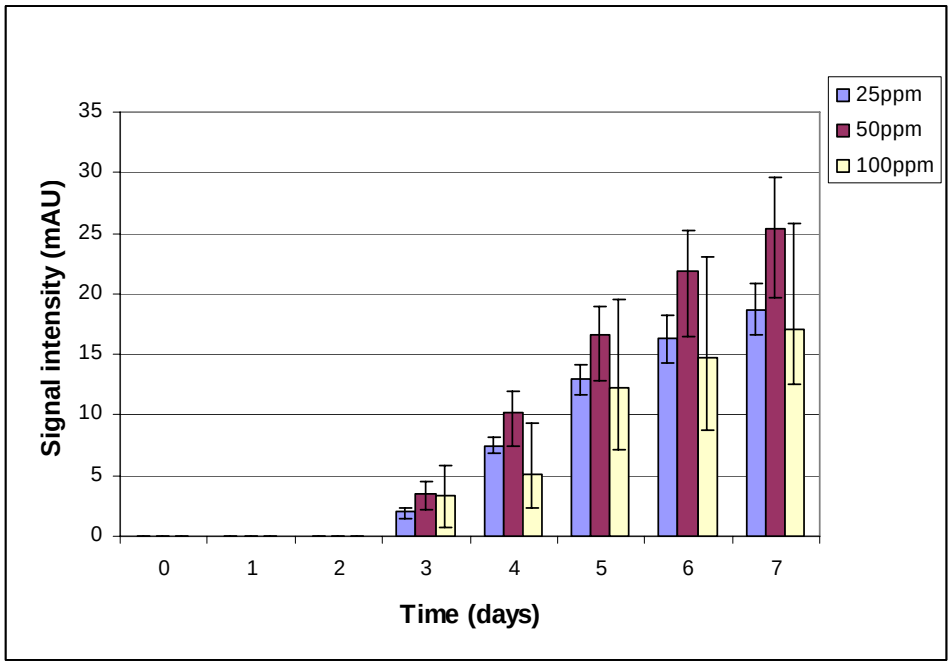


Figure 4.53 Production of 4-nitroanisole from 4-nitroaniline with different initial concentrations

Surprisingly, neither 25 ppm or 100 ppm starting 4-nitroaniline concentrations showed the highest level for production of 4-nitroanisole (Figure 4.53). However, when the complexity of production of 4-nitroanisole is taken into consideration, such results may be understood. A higher starting concentration can produce more 4-nitrophenol in the culture, which provides more 4-nitrophenol available to transform into 4-nitroanisole. Meanwhile, a higher starting concentration of 4-nitroaniline would reach a lower level of degradation. Therefore, the way to produce 4-nitroanisole directly from 4-nitroaniline was depressed. Thus a middle ground could be reached with a middle level of starting concentration of 4-nitroaniline.

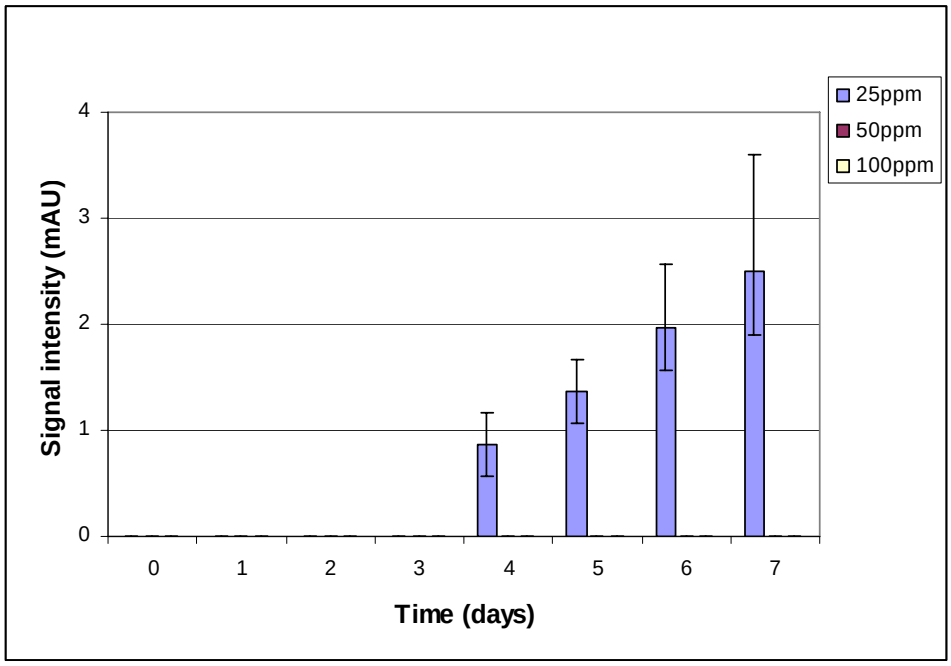


Figure 4.54 Production of 1,2-dimethxoy-4-nitrobenzene from 4-nitroaniline with different initial concentrations

The mechanism of the production of 1,2-dimethoxy-4-nitrobenzene is not yet clearly understood. In this study, it was detected in different degradation cultures, including the ones with dyes and ones with 4-nitroaniline and 4-nitroanisole. In the case of 4-nitroaniline (Figure

4.54), only in 25 ppm starting concentration cultures was 1,2-dimethoxy-4-nitrobenzene detected. Other research has shown 1,2-dimethoxy-4-nitrobenzene can be completely mineralized by another type of white rot fungus *Phanerochaete chrysosporium*, into nitrite, water and CO_2 (Valli, K et al., 1992).

Since several secondary products have been discovered from degradations of two of the three primary products, the question may be asked as to whether further products will be formed from the degradation of the secondary products. In order to answer this question, experiments were carried out to find any possible products from the degradations of the secondary products. The fungal cultures were prepared with 10 mM buffer concentration and incubated for 3 days before the addition of individual secondary products to give an initial concentration of 25ppm. After 4 days degradation, the cultures were sampled and analyzed by GC/MS and HPLC. Four days after the addition of 4-nitroanisole, veratryl alcohol and 4-nitrophenol were detected. In the gas chromatogram, the peak at 17.481 min was assigned to 4-nitrophenol (Figure 4.55). The peak at 25.376 min in HPLC chromatogram also confirmed that 4-nitrophenol was produced (Figure 4.56). The unknown peak at 29.555 min was not 1,2-dimethoxy-4-nitrobenzene, even though the retention time was very close to that of 1,2-dimethoxy-4-nitrobenzene. However, the UV spectrum of that peak was totally different from that of 1,2-dimethoxy-4-nitrobenzene. The production of 4-nitrophenol suggested that the reverse reaction of methylation, demethylation of 4-nitroanisole into 4-nitrophenol did exist. Such demethylation has also been reported by Valli et al, (1992) in research of using *Phanerochaete chysosporium* to degrade 2,4-dinitrotoluene.

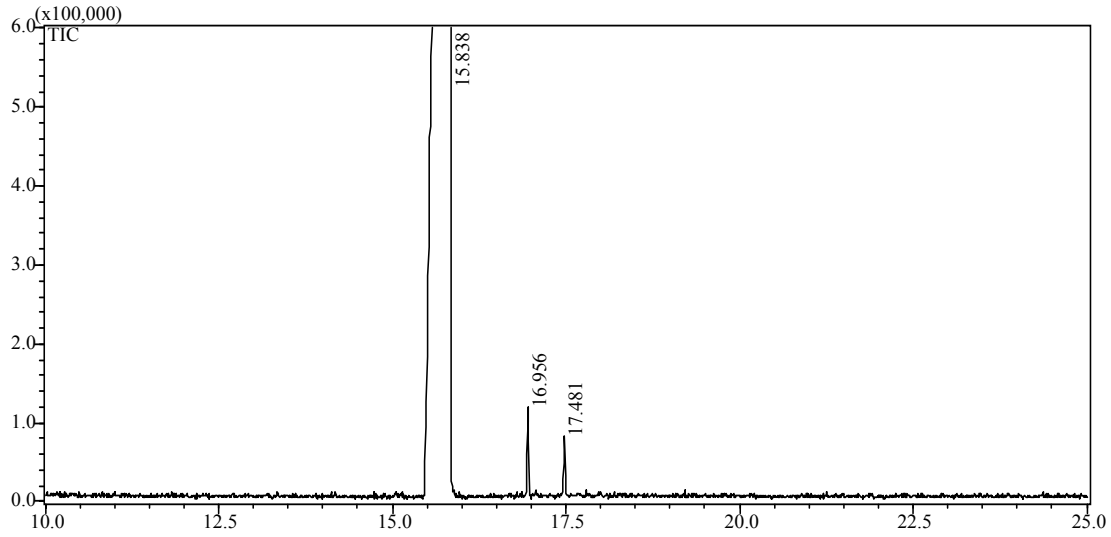


Figure 4.55 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 4 day culture having 4-nitroanisole — 4-nitroanisole (15.838 min), veratryl alcohol (16.956 min), 4-nitrophenol (17.481 min)

Four days after 4-nitroguaiacol was added into culture, 1,2-dimethoxy-4-nitrobenzene was detected (Figure 4.57). Gas chromatogram clearly showed the peak of 1,2-dimethoxy-4-nitrobenzene at 18.369 min, and the peak at 29.609 min in HPLC chromatogram was also assigned to 1,2-dimethoxy-4-nitrobenzene (Figure 4.58). This result has already been reported in earlier (Lu, 2004). Therefore, to this point, 1,2-dimethoxy-4-nitrobenzene has been detected in various degradation cultures, which apparently indicates that there are more than one mechanism involved in the production of 1,2-dimethoxy-4-nitrobenzene. Nevertheless, when 1,2-dimethoxy-4-nitrobenzene was added to cultures as the initial compound, no further degradation products were found (Figure 4.59). Gas chromatogram showed only two more compounds, veratryl alcohol and veratryl aldehyde. But this result did not eliminate the likelihood that 1,2-dimethoxy-4-nitrobenzene was further degraded by *Pleurotus ostreatus*. GC/MS is only able to identify volatile or semi-volatile compounds. In this case, if 1,2-dimethoxy-4-nitrobenzene was degraded into compounds that are not volatile, then these compounds would not be seen in the

gas chromatogram. HPLC analysis was not conducted for the degradation of 1,2-dimethoxy-4-nitrobenzene due to its lack of capability of identifying unknown compounds. A HPLC system coupled with mass spectrometry will be very useful for future study.

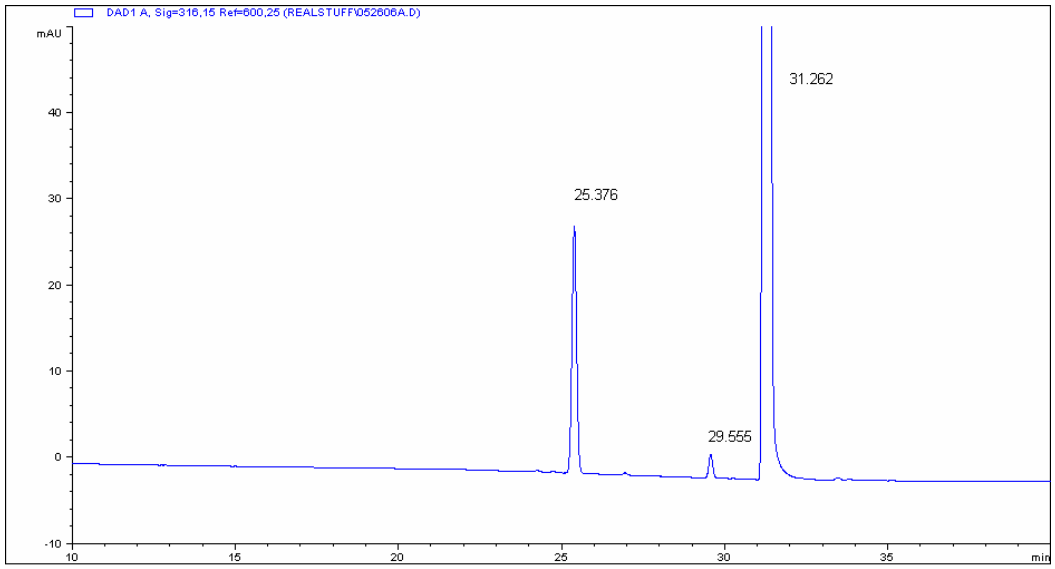


Figure 4.56 HPLC chromatogram of 4 day culture having 4-nitroanisole (316nm) — 4-nitrophenol (25.376min), unknown metabolite (29,555min), 4-nitroanisole (31.262min).

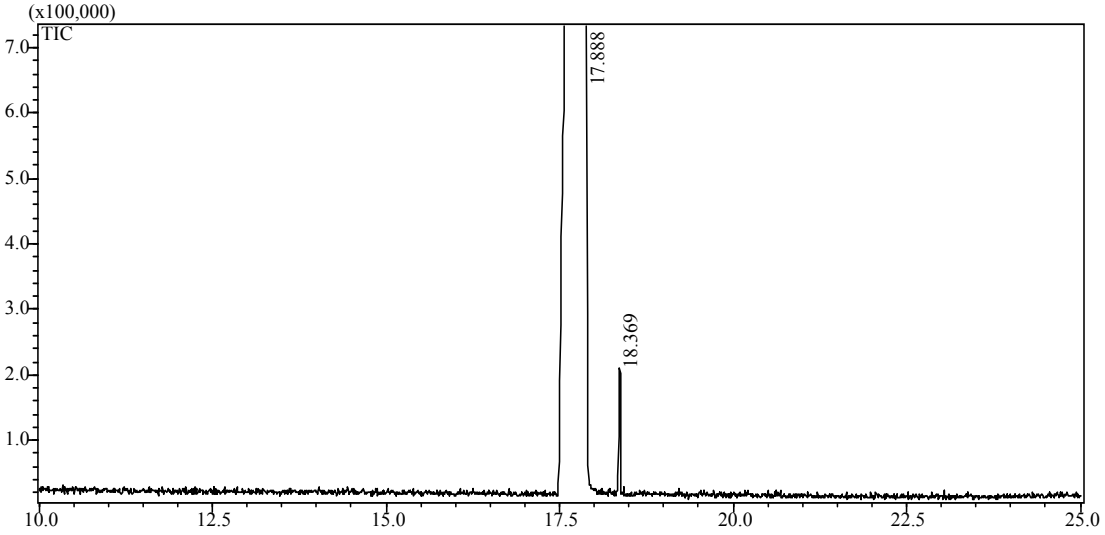


Figure 4.57 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 4 day culture having 4-nitroguaiacol — 4-nitroguaiacol (17.888min), 1,2-dimethoxy-4-nitrobenzene (18.369min)

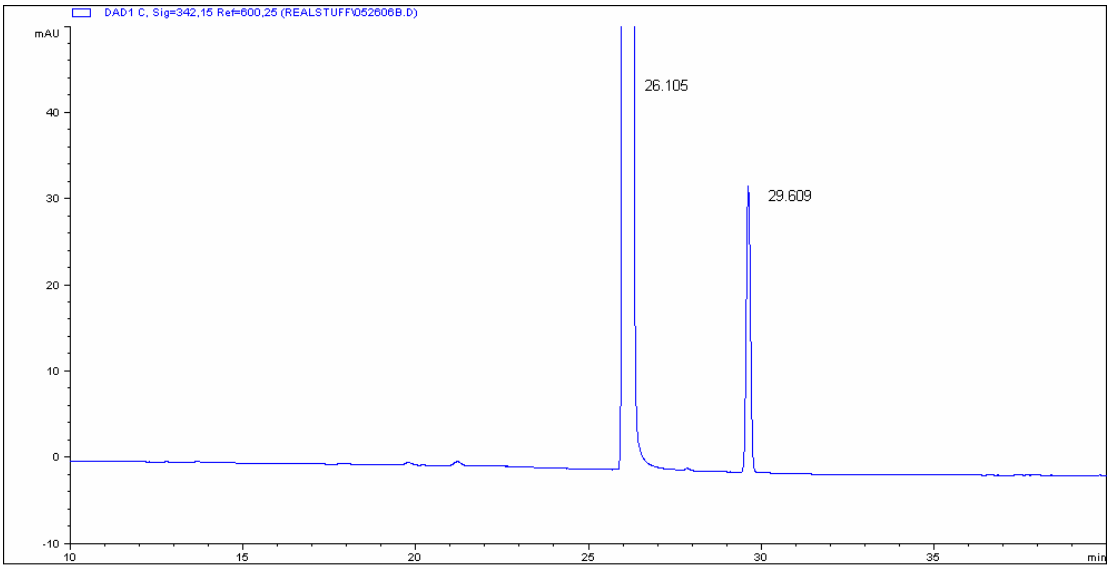


Figure 4.58 HPLC chromatogram of 4 day culture having 4-nitroguaiacol (316nm) — 4-nitroguaiacol (26.105min), 1,2-dimethoxy-4-nitrobenzene (29.609min)

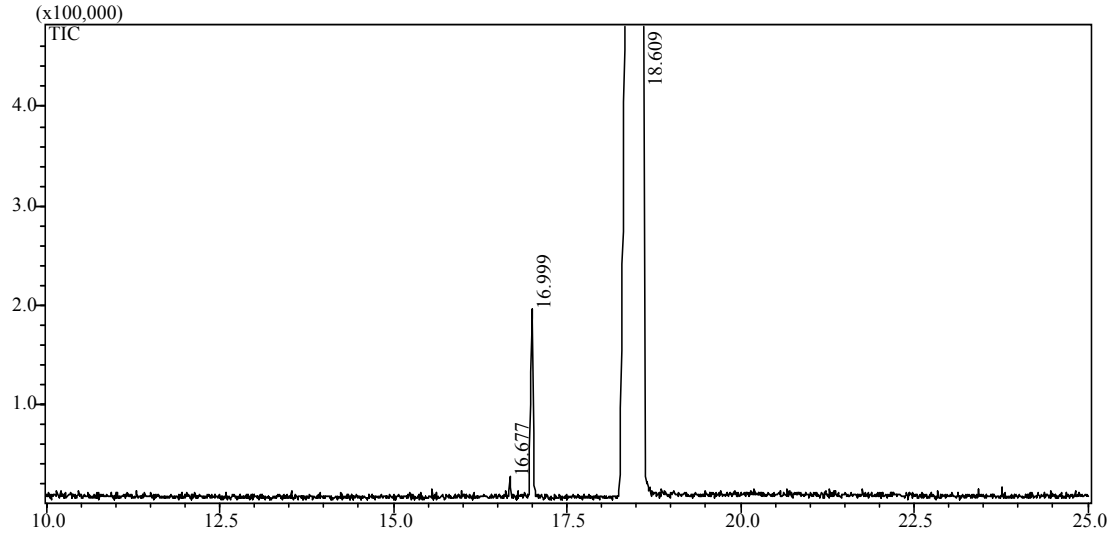


Figure 4.59 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 4 day culture having 1,2-dimethoxy-4-nitrobenzene — veratryl aldehyde (16.677 min), veratryl alcohol (16.999 min), 1,2-dimethoxy-4-nitrobenzene (18.609 min)

It was discussed that the 2,4-dinitrophenol may be undergoing a methylation step and transform into 2,4-dinitroanisole. However, the gas chromatograms of 4 day cultures of 2,4-dinitrophenol and 2,4-dinitroanisole did not support such a suggestion (Figure 4.60). No 2,4-dinitroanisole was detected in the culture initially having 2,4-dinitrophenol. One and two unknown compounds were detected for 2,4-dinitrophenol and 2,4-dinitroanisole (Figure 4.61), respectively, and the structures of these new compounds were not clear. As was previously shown, the concentration of the initial compounds added to culture can play a critical role in degradation. In this case, a 25 ppm starting concentration of 2,4-dinitrophenol may or may not be too high for the methylation to happen. Further research needs to be done to study that.

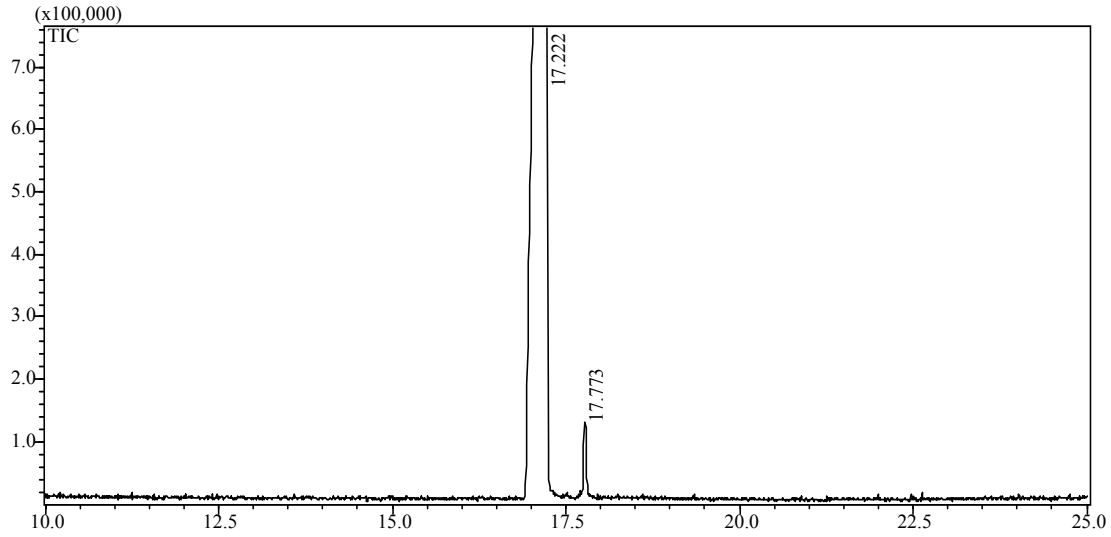


Figure 4.60 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 4 day culture having 2,4-dinitrophenol — 2,4-dinitrophenol (17.222 min), unknown metabolite (17.773 min).

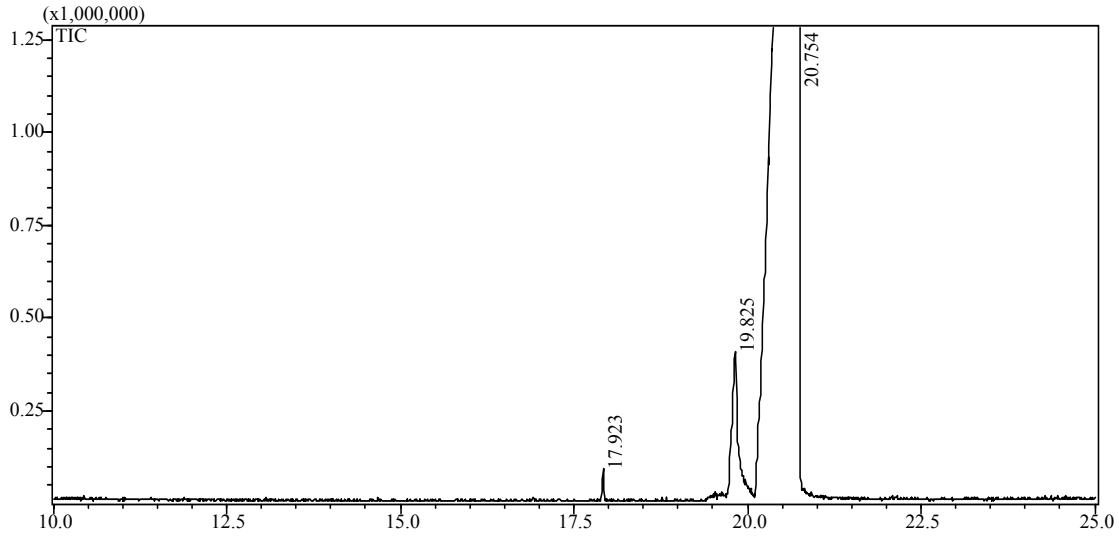


Figure 4.61 X axis (retention time), Y axis (signal intensity). Gas chromatogram of 4 days culture having 2,4-dinitroanisole — unknown metabolites (17.923 min and 19.825 min), 2,4-dinitroanisole (20.754 min)

Given the degradation results of 4-nitrophenol and its descendants, a scheme of the degradation pathway of 4-nitrophenol was proposed (Figure 4.62). The 4-nitroanisole appeared to be one of the major products from 4-nitrophenol, and it was also able to revert back to 4-nitrophenol through a demethylation step. The products 2,4-dinitrophenol and 4-nitroguaiacol seemed to be produced by two competing mechanisms With a low starting 4-nitrophenol concentration, the production of 2,4-dinitrophenol was favored. A starting concentration of 4-nitrophenol as high as 50ppm was more likely to produce 4-nitroguaiacol.

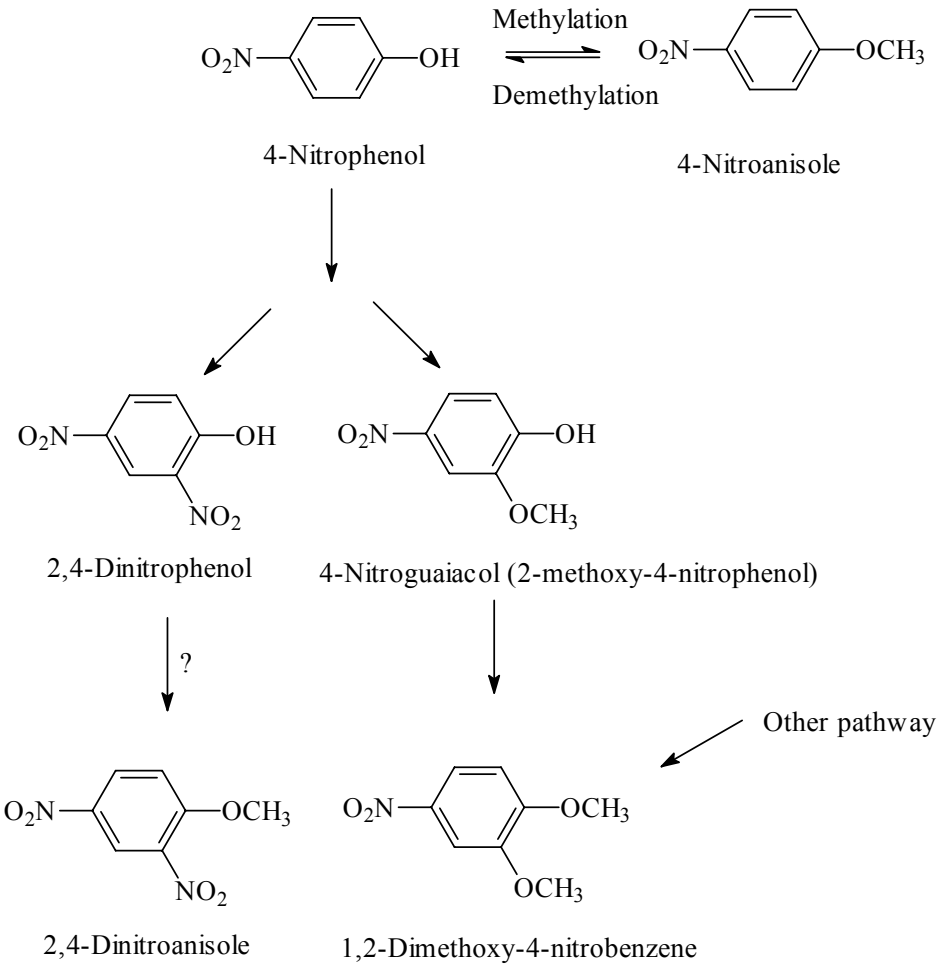


Figure 4.62 A scheme of the degradation pathway of 4-nitrophenol

The 4-nitroguaiacol was found to transform into 1,2-dimethoxy-4-nitrobenzene, but whether a similar methylation step is responsible for the production of 2,4-dinitroanisole is still not clear. The 1,2-dimethoxy-4-nitrobenzene has been found to be produced by cultures initially having different compounds, including dyes, 4-nitrophenol, 4-nitroaniline and 4-nitroguaiacol. It was suggested that more than one mechanism was responsible for the production of 1,2-dimethoxy-4-nitrobenzene. The mechanisms involved in the production of 1,2-dimethoxy-4-nitrobenzene need to be studied in the future.

CHAPTER 5

CONCLUSION AND FURTHER DISCUSSION

This study investigated the degradation of five nitrophenylazophenylamine dye by white rot fungus *Pleurotus ostreatus*. The effects of buffer concentration, pH, agitation and temperature on decolorization of three water soluble dyes, Orange II, Acid Red 29 and Remazol Brilliant Blue R were studied to establish the optimal conditions for decolorization. Such conditions were then applied in the degradation of Disperse Red 1, Disperse Red 19, Disperse Orange 1, Disperse Orange 25 and Disperse Red 13. The primary products from these five azo dyes were identified by GC/MS and HPLC. Further degradation of the primary products was also investigated. The degradation pathways of these dyes were proposed, and the degradation pathways of the primary products were also discussed. The major findings of this study are summarized as follows.

1. Using the water soluble dyes, the buffer concentration of the fungal culture was found to play a significant role in fungal growth and subsequent decolorization. Without buffer, the cultures were not able to decolorize anyone of the three water soluble dyes. However, a buffer concentration of 30 mM was also not satisfactory for decolorization. The optimal buffer concentration was found at 10 mM or 20 mM.
2. The initial pH value of the fungal culture also was found to be critical for decolorization. An initial pH at 3 or 4 did not give significant decolorization,

while pH at 5 or 7 seemed to be the most suitable for decolorization of all three water soluble dyes.

3. Agitation was essential for the decolorization of the three water soluble dyes. Without agitation, no decolorization was observed. However, with agitation at 150 rpm, substantial decolorization was seen.

4. The effect of culture temperature on decolorization was examined. Both 30 ºC and 35 ºC gave high efficiency of decolorization of Orange II. The 25 ºC condition though lagging behind 30 ºC and 35 ºC, achieved a high degree of decolorization after 3 days.

5. All three water soluble dyes showed some extent of bio-adsorption, 20 percent at most, on dead fungal mass. However, this amount of bio-adsorption contributes only a small portion of the decolorization observed in the live fungal cultures.

6. Ten mM buffer, pH of 5 and 30 ºC seemed to be the optimal condition for decolorization of the water.

7. Nitrobenzene, 4-nitrophenol and 4-nitroaniline were identified as the three primary products from Disperse Red 1, Disperse Red 19, Disperse Orange 1, Disperse Orange 25. A degradation pathway of these four structurally-similar dyes was discussed. Disperse Red 13 was found to produce 1-chloro-3-nitrobenzene, 2-chloro-4-nitroanisole and 2-chloro-4-nitroaniline as its degradation products.

8. No further degradation products of nitrobenzene were identified.

9. Five compounds, 4-nitroanisole, 1,2-dinitrophenol, 1,2-dintroanisole, 4-nitroguaiacol and 1,2-dimethoxy-4-nitrobenzene were detected in the culture initial containing 4-nitrophenol. A degradation pathway of 4-nitrophenol was proposed. The initial concentration of the 4-nitrophenol added to fungal cultures was found to be crucial for degradation. A concentration of 100 ppm was apparently too toxic and inhibited degradations. Given a concentration of 25 ppm, the fungal culture was able to degrade more than 90 percent of 4-nitrophenol within 7 days.

10. Four compounds, nitrobenzene, 4-nitrophenol, 4-nitroanisole and 1,2-dimethoxy-4-nitrobenzene, were detected in the culture initially containing 4-nitroaniline after 7 days. A degradation pathway of 4-nitroaniline was proposed. The initial concentration of the 4-nitroaniline added to the fungal cultures was found to affect degradation, however, not as much as in the case of 4-nitrophenol.

11. The product 1,2-dimethoxy-4-nitrobenzene has been found to be produced by cultures initially having various compounds, including dyes, 4-nitrophenol, 4-nitroaniline and 4-nitroguaiacol. It was suggested that more than one mechanism was responsible for the production of 1,2-dimethoxy-4-nitrobenzene.

The optimum culture parameters, including buffer concentration, pH, agitation and temperature were established from decolorization of three water soluble dyes, which were then used for the degradation of five azo dyes. However, enzyme productions and their possible correlations with decolorization efficiency were still not successfully determined. In future

work, more study has to be done on the enzyme production and other factors involved in the decolorization and degradation.

Primary degradation products from the five nitrophenylazophenylamine dyes were identified. The proposed pathway of such degradations may be applied to structurally similar dyes in other studies. Several secondary products were discovered from the primary products. However, our current analytical techniques may not be able to identify all possible degradation products. GC/MS is only able to detect volatile or semi-volatile compounds, while HPLC is not quite powerful in identifying unknown compounds without Mass spectrometry. Techniques like LC/MS may be needed in future study. Other techniques may also be applied to answer whether complete mineralization happens to dye in the degradation.

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APPENDICES

Part 1. Data for decolorization of three water soluble dyes with different buffer concentration and initial pH values.

Change in pH for Orange II decolorization with different buffer concentrations

		Day(s)										
		0	1	2	3	4	5	6	7	8	9	10
No buffer	Mean	5.51	5.42	3.99	3.28	3.24	3.22	3.26	3.26	3.28	3.24	3.26
10mM buffer	Mean	5.00	5.04	5.46	3.93	5.08	5.40	4.97	4.62	4.84	5.14	5.17
20mM buffer	Mean	5.00	5.05	5.15	5.02	4.76	4.91	5.13	5.09	5.41	5.23	4.89
30mM buffer	Mean	5.00	5.03	5.07	5.39	5.42	5.24	5.27	5.22	5.11	5.40	5.53

Effect of buffer concentration on Orange II decolorization (color remaining %)

		Day(s)							
		0	1	2	3	4	5	6	7
No buffer	Mean	100.00	100.00	100.00	99.82	99.23	97.87	98.75	97.25
	Error +	0.00	0.00	0.00	0.81	0.54	1.34	0.61	0.28
	Error -	0.00	0.00	0.00	0.81	0.54	1.34	0.61	0.28
10mM buffer	Mean	100.00	90.46	43.65	10.28	9.39	7.63	6.81	5.31
	Error +	0.00	0.40	1.09	0.03	0.20	0.27	0.00	0.10
	Error -	0.00	0.40	1.09	0.03	0.20	0.27	0.00	0.10
20mM buffer	Mean	100.00	83.23	42.55	10.32	9.74	7.98	6.28	4.94
	Error +	0.00	0.30	1.82	0.08	0.14	0.20	0.30	0.30
	Error -	0.00	0.30	1.82	0.08	0.14	0.20	0.30	0.30
30mM buffer	Mean	100.00	100.00	79.56	41.24	9.85	8.34	9.50	9.97
	Error +	0.00	0.00	1.08	5.03	1.11	0.21	0.09	0.03
	Error -	0.00	0.00	1.08	5.03	1.11	0.21	0.09	0.03

Change in pH for Acid Red 29 decolorization with different buffer concentrations
Dye was added to the culture on 3rd day

		Day(s)										
		0	1	2	3	4	5	6	7	8	9	10
No buffer	Mean	5.51	5.31	3.78	3.46	3.31	3.28	3.24	3.25	3.24	3.23	3.23
10mM buffer	Mean	5	4.98	6.58	4.22	4.69	4.60	4.70	4.69	4.64	4.51	4.54
20mM buffer	Mean	5	4.97	5.19	5.04	4.54	4.64	4.69	4.67	4.64	4.59	4.59
30mM buffer	Mean	5	4.97	5.045	5.335	5.225	4.905	4.855	5.12	5.57	5.23	4.77

Effect of buffer concentration on Acid Red 29 decolorization (color remaining %)

		Day(s)							
		0	1	2	3	4	5	6	7
No buffer	Mean	100.0	99.8	99.9	99.7	98.5	99.6	100.0	99.7
	Error +	0.0	0.9	0.5	0.9	0.0	0.6	0.8	0.6
	Error -	0.0	0.9	0.5	0.9	0.0	0.6	0.8	0.6
10mM buffer	Mean	100.0	53.7	25.5	20.1	17.3	15.3	13.6	12.8
	Error +	0.0	8.8	1.5	1.2	0.6	0.7	0.6	0.6
	Error -	0.0	8.8	1.5	1.2	0.6	0.7	0.6	0.6
20mM buffer	Mean	100.0	67.2	39.2	25.1	22.5	21.5	22.1	21.7
	Error +	0.0	0.4	0.1	0.0	0.6	0.7	0.8	0.2
	Error -	0.0	0.4	0.1	0.0	0.6	0.7	0.8	0.2
30mM buffer	Mean	100.0	99.7	95.4	67.5	68.7	69.9	70.3	69.6
	Error +	0.0	0.1	0.9	1.2	1.3	1.3	1.3	1.3
	Error -	0.0	0.1	0.9	1.2	1.3	1.3	1.3	1.3

Change in pH for RBBR decolorization with different buffer concentrations
Dye was added to the culture on 3rd day

		Day(s)								
		0	1	2	3	4	5	6	7	8
No buffer	Mean	5.50	5.25	3.61	3.22	3.29	3.21	3.29	3.28	3.27
10mM buffer	Mean	5.00	5.02	6.40	3.82	4.84	4.54	4.78	4.67	4.76
20mM buffer	Mean	5.00	5.01	5.24	4.68	4.68	4.83	4.79	4.67	4.65
30mM buffer	Mean	5.00	5.02	5.12	5.54	4.93	4.80	5.17	5.25	5.02

Effect of buffer concentration on RBBR decolorization (color remaining %)

		Day(s)					
		0	1	2	3	4	5
No buffer	Mean	100.0	96.5	96.5	97.5	98.2	97.0
	Error +	0.0	0.8	0.3	0.1	0.6	0.9
	Error -	0.0	1.4	0.5	0.1	0.4	1.5
10mM buffer	Mean	100.0	99.3	90.9	59.6	37.9	32.6
	Error +	0.0	0.7	2.9	12.0	0.6	0.4
	Error -	0.0	0.6	2.5	8.9	0.4	0.3
20mM buffer	Mean	100.0	66.3	42.6	38.6	36.3	33.9
	Error +	0.0	4.1	1.5	1.1	1.1	1.2
	Error -	0.0	4.0	1.0	1.2	1.2	1.3
30mM buffer	Mean	100.0	97.3	80.8	81.2	83.1	81.7
	Error +	0.0	1.5	9.1	10.6	11.3	8.3
	Error -	0.0	2.0	6.4	6.2	7.2	5.9

Change in pH for Orange II decolorization with different initial pH values
Dye was added to the culture on 3rd day

		Day(s)								
		0	1	2	3	4	5	6	7	8
pH 3	Mean	3	3.14	3.11	3.13	3.10	3.01	3.00	3.02	3.02
pH 4	Mean	4	4.09	4.03	4.01	3.90	3.73	3.66	3.71	3.71
pH 5	Mean	5	5.09	7.04	5.32	5.50	5.53	5.00	4.79	4.87
pH 6	Mean	6	5.85	3.90	3.98	3.95	3.89	3.85	3.88	3.95
pH 7	Mean	7	6.77	6.11	4.32	4.87	4.81	4.88	4.76	5.33

Effect of initial pH on Orange II decolorization (color remaining %)

		Day(s)					
		0	1	2	3	4	5
pH 3	Mean	100.00	100.00	100.00	100.00	100.00	100.00
	Error +	0.00	0.00	0.00	0.00	0.00	0.00
	Error -	0.00	0.00	0.00	0.00	0.00	0.00
pH 4	Mean	100.00	99.68	99.31	98.83	99.16	99.09
	Error +	0.00	0.32	0.64	0.26	0.00	0.24
	Error -	0.00	0.32	0.64	0.26	0.00	0.24
pH 5	Mean	100.00	78.64	27.75	9.64	7.94	6.36
	Error +	0.00	0.26	0.04	0.03	0.02	0.06
	Error -	0.00	0.26	0.04	0.03	0.02	0.06
pH6	Mean	100.00	98.22	98.59	96.95	95.47	93.49
	Error +	0.00	0.49	0.29	0.87	1.47	3.45
	Error -	0.00	0.49	0.29	0.87	1.47	3.45
pH 7	Mean	100.00	71.29	30.68	9.75	8.17	6.65
	Error +	0.00	0.41	2.80	0.10	0.24	0.07
	Error -	0.00	0.41	2.80	0.10	0.24	0.07

Change in pH for Acid Red 29 decolorization with different initial pH values

		Day(s)								
		0	1	2	3	4	5	6	7	8
pH 3	Mean	3	3.08	3.09	3.00	2.88	2.78	2.81	2.78	2.85
pH 4	Mean	4	4.08	4.05	3.96	3.91	3.72	3.70	3.72	3.83
pH 5	Mean	5	5.05	6.15	3.86	4.21	4.49	4.60	4.68	4.84
pH 6	Mean	6	5.90	4.56	3.87	3.89	3.83	3.87	3.92	4.37
pH 7	Mean	7	6.91	6.66	4.19	4.19	4.39	4.77	4.91	5.46

Effect of initial pH on Acid Red 29 decolorization (color remaining %)

		Day(s)					
		0	1	2	3	4	5
pH 3	Mean	100.00	97.92	97.94	99.95	100.00	98.24
	Error +	0.00	1.03	2.06	0.05	0.00	1.76
	Error -	0.00	1.03	2.06	0.05	0.00	1.76
pH 4	Mean	100.00	99.59	99.61	90.93	76.40	69.36
	Error +	0.00	0.36	0.06	3.51	21.31	26.41
	Error -	0.00	0.36	0.06	3.51	21.31	26.41
pH 5	Mean	100.00	58.78	25.11	19.77	16.38	14.31
	Error +	0.00	8.4	2.57	1.07	1.16	0.29
	Error -	0.00	8.4	2.57	1.07	1.16	0.29
pH6	Mean	100.00	55.08	29.80	22.26	18.44	15.87
	Error +	0.00	0.25	1.06	0.18	0.01	0.34
	Error -	0.00	0.25	1.06	0.18	0.01	0.34
pH 7	Mean	100.00	71.54	23.22	17.61	14.36	13.24
	Error +	0.00	4.97	0.36	0.06	0.44	0.48
	Error -	0.00	4.97	0.36	0.06	0.44	0.48

Change in pH for RBBR decolorization with different initial pH values
Dye was added to the culture on 3rd day

		Day(s)								
		0	1	2	3	4	5	6	7	8
pH 3	Mean	3.00	3.00	3.04	3.01	2.98	2.97	2.95	2.96	2.95
pH 4	Mean	4.00	4.04	4.07	4.10	4.13	4.16	4.22	4.35	4.55
pH 5	Mean	5.00	5.02	5.24	4.65	4.65	4.87	4.90	4.84	4.75
pH 6	Mean	6.00	5.88	4.90	4.47	4.56	4.55	4.53	4.58	4.58
pH 7	Mean	7.00	6.87	6.72	4.15	4.19	4.69	4.70	4.85	5.00

Effect of initial pH on RBBR decolorization (color remaining %)

		Day(s)					
		0	1	2	3	4	5
pH 3	Mean	100	100.0	100.0	100.0	99.8	99.6
	Error +	0	0	0	0	0.2	0.5
	Error -	0	0	0	0	0.2	0.5
pH 4	Mean	100	99.9	99.7	92.1	82.3	69.5
	Error +	0	0.1	0.0	0.0	2.4	7.7
	Error -	0	0.2	0	0.3	2.5	7.7
pH 5	Mean	100	77.3	44.0	41.8	42.4	39.8
	Error +	0	0.9	0.8	1.8	1.8	1.2
	Error -	0	0.9	0.8	1.8	1.8	1.2
pH6	Mean	100	87.1	67.1	46.5	41.8	37.7
	Error +	0	2.4	2.4	0.5	0.7	0.4
	Error -	0	2.4	2.4	0.5	0.7	0.4
pH 7	Mean	100	90.3	62.6	38.5	35.7	33.4
	Error +	0	7.7	20.0	0.6	2.7	4.3
	Error -	0	7.7	20.0	0.6	2.7	4.3

Effect of agitation on decolorization (color remaining %)

		Day(s)						
		0	1	2	3	4	5	6
Orange II	Mean	100	96.7	97.2	97.2	75.0	31.9	10.5
	Error +	0	1.7	1.9	1	1.6	1.2	0.4
	Error -	0	1.2	1.5	1.2	3.7	1.9	0.3
Acid Red 29	Mean	100	98.7	99.8	99.6	97.6	49.6	26.2
	Error +	0	0.5	0.2	0.2	1.6	7.1	1.5
	Error -	0	0.7	0.4	0.2	1	10.8	1.8
RBBR	Mean	100	98.7	99.9	100.0	93.0	43.2	38.0
	Error +	0	0.2	0.1	0.0	3.9	1.6	4.2
	Error -	0	0.4	0.3	0.0	3.7	2.5	3.3

Effect of temperature on Orange II decolorization (color remaining %)

		Day(s)					
		0	1	2	3	4	5
25 ºC	Mean	100.0	99.8	82.2	28.1	11.9	11.0
	Error +	0	0.2	10.7	19.4	0.7	1.3
	Error _	0	0.4	5.8	10.7	0.7	0.7
30 ºC	Mean	100	80.5	28.2	9.5	8.1	6.6
	Error +	0	7.1	13	0.1	1	0.7
	Error -	0	6.5	11.3	0.3	0.6	0.6
35 ºC	Mean	100	86.0	41.9	9.0	7.2	6.3
	Error +	0	6.3	8.4	1	0.2	1
	Error -	0	12.1	18.2	1.2	0.1	0.9

Part 2. ANOVA analysis

ANOVA analysis results of 3rd decolorization of three water soluble dyes with different buffer concentration

	P- value and conclusion	
Orange II	4.44E-05	Significant difference due to buffer concentration
	0.00754	Significant difference between 0 and 30mM
	0.025528	Significant difference between 10M and 30mM
	0.698489	No significant difference between 10M and 20mM
Acid Red 29	1.41E-06	Significant difference due to buffer concentration
	0.000355	Significant difference between 0 and 10mM
	0.002163	Significant difference between 0 and 30mM
	0.0008	Significant difference between 20M and 30mM
	0.054135	May or may not be Significant difference between 10M and 20mM
RBBR	4.15E-05	Significant difference due to buffer concentration
	0.003713	Significant difference between 0 and 10mM
	0.028921	Significant difference between 10M and 20mM
	0.001386	Significant difference between 20M and 30mM
	0.058143	May or may not be Significant difference between 10M and 30mM

ANOVA analysis results of 3rd decolorization of three water soluble dyes with different initial pH

	P- value and conclusion	
Orange II	2.63E-10	Significant difference due to initial pH
	8.89E-06	Significant difference between pH 4 and 5
	0.000102	Significant difference between pH 6 and 7
	0.372814	No Significant difference between pH 5 and 7
Acid Red 29	0.003672	Significant difference due to initial pH
	0.383827	No significant difference between pH 3 and 4
	0.106228	No significant difference between pH 4 and 5
	0.070959	No significant difference between pH 5 and 6
	0.125566	No significant difference between pH 5 and 7
RBBR	1.64E-07	Significant difference due to initial pH
	0.001375	Significant difference between pH 4 and 5
	0.236666	No significant difference between pH 5 and 7
	0.135246	No significant difference between pH 5 and 6

ANOVA analysis of the effect of agitation

	P- value and conclusion	
Orange II	1.01E-07	Significant difference due to agitation
Acid Red 29	1.4E-07	Significant difference due to agitation
RBBR	1.08E-05	Significant difference due to agitation

ANOVA analysis of the effect of temperature

	P- value and conclusion	
All means	0.004995	Significant difference due to temperature
25 ºC vs 30 ºC	0.019119	Significant difference due to temperature
25 ºC vs 35 ºC	0.003702	Significant difference due to temperature
30 ºC vs 35 ºC	0.300849	No significant difference due to temperature

Part 3. Quantitative analysis of degradation of Disperse Red, 4-nitrophenol and 4-nitroaniline with different initial concentrations.

Production of Nitrobenzene from Disperse Red 1 (mAU)

		Day(s)							
		0	1	2	3	4	5	6	7
50 ppm	Mean	0.0	3.3	87.1	168.4	238.7	313.4	329.9	311.8
	Error +	0.0	3.4	30.4	21.5	24.1	36.5	20.8	25.4
	Error -	0.0	1.7	36.6	35.3	51.1	49.6	21.7	26.5
100 ppm	Mean	0.0	19.3	107.2	204.3	249.8	283.3	314.7	372.0
	Error +	0.0	30.1	57.5	49.6	54.3	52.9	58.2	92.2
	Error -	0.0	15.3	31.8	25.4	34.3	36.0	46.2	87.6
200 ppm	Mean	0.0	12.2	70.3	197.2	235.3	266.9	292.8	355.6
	Error +	0.0	9.0	45.4	12.7	18.6	19.0	37.9	32.3
	Error -	0.0	4.9	29.8	21.3	10.7	24.6	20.3	39.1

Production of 4-nitrophenol from Disperse Red 1 (mAU)

		Day(s)							
		0	1	2	3	4	5	6	7
50 ppm	Mean	0.0	3.5	67.6	112.6	105.2	54.5	33.7	29.0
	Error +	0.0	3.1	21.3	6.5	5.4	20.8	7.6	7.8
	Error -	0.0	1.8	29.0	15.6	3.5	22.7	4.4	4.9
100 ppm	Mean	0.0	16.8	80.0	165.6	188.8	190.4	156.7	137.8
	Error +	0.0	25.2	39.5	20.3	9.4	19.0	31.3	28.8
	Error -	0.0	12.9	23.1	15.5	11.2	20.2	15.9	21.8
200 ppm	Mean	0.0	12.0	51.8	167.5	188.4	217.4	240.0	226.9
	Error +	0.0	7.2	40.9	22.3	17.0	10.5	30.9	54.4
	Error -	0.0	4.0	25.5	17.8	13.0	13.4	44.6	61.4

Production of 4-nitroaniline from Disperse Red 1 (mAU)

		Day(s)							
		0	1	2	3	4	5	6	7
50 ppm	Mean	0.0	21.0	50.5	59.6	57.2	46.4	32.2	24.6
	Error +	0.0	6.5	2.3	13.4	11.8	18.1	12.1	5.1
	Error -	0.0	5.1	3.2	10.1	9.1	16.9	13.2	8.9
100 ppm	Mean	0.0	28.4	61.9	77.6	85.5	90.3	84.8	74.7
	Error +	0.0	11.0	4.6	6.8	6.7	10.0	10.4	5.9
	Error -	0.0	7.9	8.3	4.8	12.8	16.9	11.7	3.2
200 ppm	Mean	0.0	36.9	83.8	103.4	121.3	140.3	143.9	133.4
	Error +	0.0	12.9	28.7	25.4	14.2	8.5	14.6	14.4
	Error -	0.0	6.6	13.3	20.5	15.5	11.7	10.6	22.1

Production of 4-nitroanisole from Disperse Red 1 (mAU)

		Day(s)							
		0	1	2	3	4	5	6	7
50 ppm	Mean	0.0	0.0	9.8	28.7	73.9	100.8	84.5	56.2
	Error +	0.0	0.0	5.2	9.0	16.7	20.5	19.0	15.8
	Error -	0.0	0.0	4.8	7.6	20.5	17.1	27.3	23.9
100 ppm	Mean	0.0	1.0	13.5	33.4	59.3	87.5	125.6	163.8
	Error +	0.0	1.8	9.3	23.3	28.9	52.8	55.3	32.4
	Error -	0.0	1.0	6.0	13.4	21.5	35.5	34.5	28.6
200 ppm	Mean	0.0	0.0	8.8	25.8	47.7	68.7	91.5	134.8
	Error +	0.0	0.0	1.8	12.0	5.8	2.8	31.1	38.4
	Error -	0.0	0.0	1.9	8.3	5.8	2.9	21.3	37.3

Reduction of 4-nitrophenol (% remaining)

		Day(s)							
		0	1	2	3	4	5	6	7
25 ppm	Mean	100	97.2	91.3	60.8	27.6	8.3	3.3	0.9
	Error +	0	0.1	1.6	3.7	2.3	2.1	1.5	0.4
	Error -	0	0.2	1.2	3.6	2.7	2.2	0.9	0.3
50 ppm	Mean	100	99.8	98.4	97.5	95.5	90.3	91.4	89.3
	Error +	0	0.2	1.2	2	3.3	8.5	7.6	8.7
	Error -	0	0.5	1	1	2.3	7.7	4.9	6.1
100 ppm	Mean	100	100.0	100.0	100.0	99.7	100.0	100.0	100.0
	Error +	0	0	0	0	0.1	0	0	0
	Error -	0	0	0	0	0.1	7.7	0	0

Production of 4-nitroanisole from 4-nitrophenol (mAU)

		Day(s)							
		0	1	2	3	4	5	6	7
25 ppm	Mean	0	143.5	358.2	615.7	887.3	1019.7	1062.8	1047.4
	Error +	0	5.9	21.8	52.6	102.7	85	66.8	46.9
	Error -	0	7.3	34.7	81.2	143.5	127	92.2	88.3
50 ppm	Mean	0	54.1	160.9	286.0	450.8	583.3	707.1	868.5
	Error +	0	39.4	47.2	152.9	291.9	501.1	741	982.5
	Error -	0	22.6	93.9	191.8	334.5	447	551	697
100 ppm	Mean	0	0	0	0	0	0	0	0
	Error +	0	0	0	0	0	0	0	0
	Error -	0	0	0	0	0	0	0	0

Production of 1,2-dinitrophenol from 4-nitrophenol (mAU)

		Day(s)							
		0	1	2	3	4	5	6	7
25 ppm	Mean	0	0.0	12.7	231.8	417.4	509.8	448.8	385.1
	Error +	0	0.0	6.9	6.8	8.8	41.1	15.9	15.2
	Error -	0	0.0	11.0	6.4	7.5	24.3	10.2	22.1
50 ppm	Mean	0	0	0	0	0	0	0	0
	Error +	0	0	0	0	0	0	0	0
	Error -	0	0	0	0	0	0	0	0
100 ppm	Mean	0	0	0	0	0	0	0	0
	Error +	0	0	0	0	0	0	0	0
	Error -	0	0	0	0	0	0	0	0

Production of 1,2-dinitroanisole from nitrophenol (mAU)

		Day(s)							
		0	1	2	3	4	5	6	7
25 ppm	Mean	0	0.0	0.0	12.0	39.8	79.3	113.0	148.4
	Error +	0	0.0	0.0	5.2	5.7	6.1	5.3	7.1
	Error -	0	0.0	0.0	3.2	6.3	10.1	9.4	10.2
50 ppm	Mean	0	0	0	0	0	0	0	0
	Error +	0	0	0	0	0	0	0	0
	Error -	0	0	0	0	0	0	0	0
100 ppm	Mean	0	0	0	0	0	0	0	0
	Error +	0	0	0	0	0	0	0	0
	Error -	0	0	0	0	0	0	0	0

Production of 1,2-dimethoxy-4-nitrobenzene from 4-nitrophenol (mAU)

		Day(s)							
		0	1	2	3	4	5	6	7
25 ppm	Mean	0	0.5	2.2	2.3	5.0	9.9	11.2	13.5
	Error +	0	0.2	0.1	0.2	1.2	1.8	0.3	0.7
	Error -	0	0.5	0.2	0.2	0.7	2.1	0.5	0.9
50 ppm	Mean	0	0.0	1.1	0.6	6.6	10.3	14.1	18.7
	Error +	0	0	1.5	1.1	4.6	8.1	12.9	18.6
	Error -	0	0	1.1	0.6	5.6	9.9	12.3	16.2
100 ppm	Mean	0	0	0	0	0	0	0	0
	Error +	0	0	0	0	0	0	0	0
	Error -	0	0	0	0	0	0	0	0

Production of 4-nitroguaiacol from 4-nitrophenol (mAU)

		Day(s)							
		0	1	2	3	4	5	6	7
25 ppm	Mean	0	3.5	0.0	0.0	0.0	0.0	0.0	0.0
	Error +	0	0.2	0.0	0.0	0.0	0.0	0.0	0.0
	Error -	0	0.2	0.0	0.0	0.0	0.0	0.0	0.0
50 ppm	Mean	0	5.4	9.3	11.1	12.7	12.9	14.2	15.0
	Error +	0	1.2	2.4	2.1	1.5	1.1	1.4	2
	Error -	0	1.4	1.9	2.3	1.8	1.4	1.7	2.9
100 ppm	Mean	0	0	0	0	0	0	0	0
	Error +	0	0	0	0	0	0	0	0
	Error -	0	0	0	0	0	0	0	0

Reduction of 4-nitroaniline (% remaining)

		Day(s)							
		0	1	2	3	4	5	6	7
25 ppm	Mean	100	98.0	88.7	75.7	63.9	53.6	45.1	37.1
	Error +	0	1.1	2.4	1.3	7.1	2.4	4	5
	Error -	0	0.9	1.4	1	7.6	3.2	5.1	6.2
50 ppm	Mean	100	98.4	91.6	82.8	74.2	67.2	61.7	57.5
	Error +	0	0.7	2.7	4.2	5.7	7.1	8.5	9.8
	Error -	0	0.4	1.7	2.8	5.5	8.1	10.6	12.7
100 ppm	Mean	100	98.2	95.0	91.0	87.2	83.8	80.7	78.5
	Error +	0	0.5	0.8	1.1	1.5	1.8	2	2.2
	Error -	0	0.7	1.6	1.9	2.7	3	2.9	2.9

Production of nitrobenzene from 4-nitroaniline (mAU)

		Day(s)							
		0	1	2	3	4	5	6	7
25 ppm	Mean	0	0.0	4.3	14.2	24.2	34.9	36.0	38.7
	Error +	0	0	0.3	0.9	0.8	10.4	3.5	5.0
	Error -	0	0	0.7	0.6	0.7	5.8	3.0	4.2
50 ppm	Mean	0	0.0	5.9	19.3	31.1	35.9	39.9	41.4
	Error +	0	0	1.2	3.0	8.5	7.5	11.1	13.6
	Error -	0	0	1.8	2.8	6.9	6.6	8.1	8.4
100 ppm	Mean	0	0.0	4.9	12.2	23.7	25.3	29.6	30.3
	Error +	0	0	1.7	6.2	12.6	8.0	6.8	4.7
	Error -	0	0	1.3	3.6	6.6	4.8	4.5	4.1

Production of 4-nitrophenol from 4-nitroaniline (mAU)

		Day(s)							
		0	1	2	3	4	5	6	7
25 ppm	Mean	0	0	4.5	12.9	15.1	17.2	14.7	13.8
	Error +	0	0	0.9	0.1	0.3	7.9	0.8	0.9
	Error -	0	0	0.9	0.1	0.3	6.1	1.0	0.4
50 ppm	Mean	0	0	8.0	17.3	21.8	20.9	21.2	21.5
	Error +	0	0	1.2	1.6	1.5	0.4	1.0	4.0
	Error -	0	0	2.1	1.3	0.9	0.3	1.1	3.3
100 ppm	Mean	0	0	10.6	23.1	30.3	24.4	23.2	25.0
	Error +	0	0	2.7	4.5	7.1	6.8	2.2	1.0
	Error -	0	0	2.6	4.4	8.3	5.6	1.4	1.5

Production of 4-nitroanisole from 4-nitroaniline (mAU)

		Day(s)							
		0	1	2	3	4	5	6	7
25 ppm	Mean	0	0.0	0.0	2.1	7.5	13.0	16.3	18.7
	Error +	0	0	0.0	0.3	0.6	1.1	1.9	2.1
	Error -	0	0	0.0	0.6	0.7	1.4	2.0	2.1
50 ppm	Mean	0	0.0	0.0	3.5	10.2	16.7	21.8	25.4
	Error +	0	0	0.0	1.0	1.7	2.3	3.4	4.3
	Error -	0	0	0.0	1.3	2.8	3.8	5.4	5.7
100 ppm	Mean	0	0.0	0.0	3.3	5.2	12.3	14.8	17.1
	Error +	0	0	0.0	2.5	4.1	7.3	8.2	8.8
	Error -	0	0	0.0	2.5	2.8	5.1	6.0	4.5

Production of 1,2-dimethoxy-4-nitrobenzene from 4-nitroaniline (mAU)

		Day(s)							
		0	1	2	3	4	5	6	7
25 ppm	Mean	0	0	0	0	0.9	1.4	2.0	2.5
	Error +	0	0	0	0	0.3	0.3	0.6	1.1
	Error -	0	0	0	0	0.3	0.3	0.4	0.6
50 ppm	Mean	0	0	0	0	0	0	0	0
	Error +	0	0	0	0	0	0	0	0
	Error -	0	0	0	0	0	0	0	0
100 ppm	Mean	0	0	0	0	0	0	0	0
	Error +	0	0	0	0	0	0	0	0
	Error -	0	0	0	0	0	0	0	0