Application of White-rot Fungi for the Biodegradation of Natural Organic Matter in Wastes

A thesis submitted in the fulfilment of the requirements for the degree of Master of Engineering

Monn Kwang Lee
Bachelor of Engineering (Chemical Engineering)

School of Civil and Chemical Engineering
RMIT University, Melbourne

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Declaration

I, Monn Kwang Lee, certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

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Abbreviations

A<sub>254</sub>  Absorbance at 254 nm
A<sub>446</sub>  Absorbance at 446 nm
AOC  Assimilable organic carbon
AWQC  Australian Water Quality Centre
BDOC  Biodegradable dissolved organic carbon
BOM  Biodegradable organic matter
CHA  Hydrophilic charged
C:N  Ratio of carbon to nitrogen
DBP  Disinfection by-products
DMP  2, 6-dimethoxyphenol
DNS  3', 5'-dinitrosalicylic acid
DOC  Dissolved organic carbon
GAC  Granular activated carbon
HAAs  Haloacetic acids
HPSEC  High performance size exclusion chromatography
Lac  Laccase
LAS  Linear alkyl benzene sulphonate
LiP  Lignin peroxidase
M<sub>i</sub>  Molecular weight
M<sub>n</sub>  Number average molecular weight,  \( M_n = \frac{\Sigma n_i M_i}{\Sigma n_i} \)
M<sub>w</sub>  Weight average molecular weight,  \( M_w = \frac{\Sigma n_i M_i^2}{\Sigma n_i M_i} \)
M<sub>w</sub>: M<sub>n</sub>  Polydispersity
MEA  Malt extract agar
MIEX<sup>TM</sup>  Magnetic ion exchange
MnP  Manganese-dependent peroxidase
n<sub>i</sub>  Number of molecules of weight M<sub>i</sub>
NEU  Hydrophilic neutral
NOM  Natural organic matter
PAC  Powdered activated carbon
PAHs  Polyaromatic hydrocarbons
PCP  Pentachlorophenol
POC  Particulate organic carbon
RBBR Remazol Brilliant Blue R
RCF  Relative centrifugal force
RH   Lignin or phenolic substrate
rpm  Revolutions per minute
SEC  Size exclusion chromatography
SHA  Slightly hydrophobic acids
SSF  Solid-state fermentation

SUVA Specific UV absorbance, $\frac{A_{254}}{DOC}$
THMs Trihalomethanes
TOC  Total organic carbon
Tween 80 Polyoxyethylene sorbitan monooleate
U    Unit
VA   Veratryl alcohol
VHA  Very hydrophobic acids
$Y_{x/s}$ Yield, $\frac{\text{biomass produced}}{\text{glucose consumption}}$
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SUMMARY

Natural organic matter (NOM), a complex mixture of organic compounds, influences drinking water quality and water treatment processes. The presence of NOM is unaesthetic in terms of colour, taste and odour, and may lead to the production of potentially carcinogenic disinfection by-products (DBPs), as well as biofilm formation in drinking water distribution systems. Some NOM removal processes such as coagulation, magnetic ion exchange resin (MIEX™) and membrane filtration produce sludge and residuals. These concentrated NOM-containing sludges from alum precipitation, membrane treatment plants and MIEX regeneration must therefore be treated prior to disposal.

The white-rot fungi possess a non-specific extracellular oxidative enzyme system composed of lignin peroxidase (LiP), manganese-dependent peroxidase (MnP) and laccase (Lac) that allows these organisms to mineralise lignin and a broad range of intractable aromatic xenobiotics. Rojek (2003) has shown the capability of Phanerochaete chrysosporium ATCC 34541 to remove 40-50% NOM from solution, however, this was found to be mainly due to adsorption and to be a partially metabolically linked activity. Consequently, the bioremediation of NOM wastes by selected white-rot fungi was further investigated in the present study.

The P. chrysosporium seemed to preferentially remove the very hydrophobic acid (VHA) fraction, and so was most effective for a NOM preparation with a high proportion of hydrophobic content (and so high in colour and specific UV absorbance (SUVA)). The extent of NOM decolourisation by P. chrysosporium in three growth media with different C:N ratios followed the trends: Waksman (C:N = 6) > Fahy (C:N = 76) > Fujita medium (C:N = 114), such that the lower the C:N ratio, the greater NOM removal. This was consistent with the findings of Rojek (2003), who used a different NOM preparation and demonstrated that the removal of NOM increased with decreased C:N ratio (1.58-15.81).

As removals of NOM with P. chrysosporium ATCC 34541 were low, and little biodegradation occurred, this organism was compared with P. chrysosporium strain ATCC 24725, Trametes versicolor ATCC 7731, and three strains of yeast (Saccharomyces species arbitrarily denoted 1, 2 and 3). T. versicolor gave the greatest removal (59%) which was attributed largely to degradation, whereas the NOM removal by the two strains of P. chrysosporium (37%) and the yeast was predominantly due to adsorption as indicated by the
deep brown colouration of the biomass. *Saccharomyces* sp. 1, 2 and 3 removed 12%, 61% and 23% of the colour, respectively. Although *Saccharomyces* sp. 2 had similar high colour reduction to *T. versicolor*, the specific removal values differed markedly: 0.055 compared to 0.089 mg NOM/mg biomass, respectively. The low level of the ligninolytic enzymes secreted by both strains of *P. chrysosporium* corresponded with the low degree of NOM removal by biodegradation as shown by high performance size exclusion chromatography (HPSEC). The high NOM removal attained by *T. versicolor* was attributed to the activities of the ligninolytic enzymes, especially laccase. The NOM removal was attributed to the breakdown of the high molecular weight compounds to form a pool of low molecular weight materials, which were then most likely utilised by the *T. versicolor*.

Growth of *T. versicolor* cultures at 36°C caused inhibition or denaturation of the activity of the phenoloxidase enzymes compared to those grown at 30°C. The low activity of LiP in both cultures suggested that this enzyme may not play much of a role in NOM removal. The higher levels of MnP and Lac activities at 30°C were responsible for the greater NOM removal (73% vs. 59%) and thus the cleavage of aromatic rings, conjugated and Cα-Cβ bonds in phenolic moieties, as well as catalysing alkyl-aryl cleavage in the NOM structures.

*T. versicolor* cultured in Waksman medium with higher initial glucose (5 g/L cf. 2 g/L) led to lower ligninolytic enzyme activities and a lower degree of NOM removal (25% less colour reduction), probably due to preferential use of glucose over NOM as carbon source. NOM removal (mg removed) increased linearly with NOM concentration up to 600 mg C/L (62 mg (A446); 31 mg (A254)), above which removal decreased markedly. This trend coincided with increasing total ligninolytic enzyme activity, where the level of Lac increased up to 600 mg C/L NOM although MnP decreased gradually across the range while LiP was only detected for 100 and 300 mg C/L NOM. Hence, the removal of NOM from solution by *T. versicolor* was associated with high oxidative enzyme activity, particularly of laccase.

Laccase was the major extracellular enzyme secreted by *T. versicolor* and by deduction, played a major role in NOM removal. The optimum temperature for Lac activity secreted by *T. versicolor* cultured in Waksman medium supplemented with 4.5 g/L wheat bran plus 0.5% Tween 80 was determined to be 50°C. The optimum pH for the Lac activity for guaiacol and NOM was identified as pH 4.0-4.5. Although the optimum enzyme activity occurred at 50°C, 30°C was recommended for enzymatic removal of NOM as the phenoloxidase enzyme activity may be denatured if the NOM removal process were considered to run for long period.
at high temperature. Although agitation led to apparent enzyme denaturation, fermentations with continuous agitation promoted enzyme activity faster than those with occasional agitation (agitated every 6 hours for 30 minutes at 130 rpm and 30°C) as it provides better mass transfer. However, it seemed that continuous agitation had an adverse effect on the fungal growth and enzyme production over extended fermentation periods.

Addition of 4.5 g/L wheat bran to modified Waksman medium in the absence of NOM led to high production of Lac activity compared with LiP and MnP activities, showing its great potential as a laccase inducer. Addition of Tween 80 alone to the cultures led to a small improvement in Lac activity; however, with the presence of wheat bran it caused marked increases in LiP, MnP and Lac activities. When NOM was added to cultures of *T. versicolor* with the two supplements, it led to markedly reduced Lac activity, but increased LiP and MnP activities, and no improvement in NOM removal compared with the cultures in the absence of supplements (12 mg (or 61%) cf. 15 mg (or 73%) for 100 mg C/L after corrected for colour from and adsorption by wheat bran).
List of Publications


Chapter 1  Introduction

The importance of maintaining high quality natural surface water sources, on both national and global scales, is well known as a major requirement for public supplies as well as for future industrial growth. The presence of natural organic matter (NOM) in raw water reduces drinking water quality and interferes with water treatment processes. NOM, a complex mixture of organic carbon compounds, is undesirable for several reasons, including its contribution to taste and odour of the water, as well as to the formation of disinfection by-products (DBPs) when NOM reacts with disinfectants such as chlorine. Many epidemiological studies have suggested that DBPs are carcinogenic and thus present a health risk to the consumer.

As disinfection remains important in public health protection because of its effectiveness in maintaining pathogen-free water and providing residual protection to control bacterial regrowth in the distribution system, its application in drinking water treatment is crucial (Carraro et al. 2000). Therefore, there has been considerable research focused on the development of more economic and efficient drinking water treatments with improved NOM removal prior to chemical disinfection. There are many NOM removal processes available, such as coagulation, granular activated carbon (GAC) adsorption, ozonation, magnetic ion exchange resin (MIEX\textsuperscript{TM}), and membrane filtration. However, these processes have been identified as having high operating cost and producing sludge and residuals, some of which contain NOM. These wastes can be problematic regarding disposal, as government legislation concerning the release of wastes has become more stringent.

Bioremediation technology is viewed as an attractive approach to the removal of NOM as this environmentally friendly method is potentially more cost-effective, and limits by-product formation and associated mutagenic product generation. This process would be able to remove biodegradable organic matter (which is responsible for microbial regrowth in the distribution systems), increase disinfectant stability in the water delivery network, as well as reduce chlorine demand (Carraro et al. 2000). Furthermore, this biological treatment could be applied in drinking water treatment as well as for the treatment of concentrated NOM wastes such as those found in alum precipitation, regenerant wastes from the MIEX\textsuperscript{TM} process, and retentates from membrane treatment plants.
Bioremediation utilises living organisms such as bacteria or fungi, or isolated enzyme systems, to break down organic pollutants and transform them into harmless products or valuable by-products (Burton et al. 1999). Previous work has shown that white-rot fungi are able to degrade lignin and a wide variety of recalcitrant organic pollutants due to their non-specific extracellular oxidative enzyme system which may include lignin peroxidase (LiP), manganese-dependent peroxidase (MnP) and laccase (Lac) (Kirk & Chang 1975, Bumpus & Aust 1987, Lonergan 1992, Barr & Aust 1994, Nyanhongo et al. 2002). *Phanerochaete chrysosporium* ATCC 34541 has been shown to remove 40-50% NOM from solution, however, this was found to be mainly due to adsorption and to be partially metabolically linked (Rojek et al. 2004). They found that environmental conditions such as carbon and nitrogen content, pH and NOM concentration played an important role in the removal of colour by the fungus. In addition, Rojek (2003) found that a combination of yeast contaminants isolated from a MIEX concentrate with the *P. chrysosporium* gave NOM removals of 70-80%.

The research herein builds on the work of Rojek (2003) and further investigates the bioremediation of NOM wastes. The aim of this research was to evaluate the effectiveness of selected white-rot fungi and yeast for the biodegradative removal of concentrated NOM from solution. The effectiveness of the white-rot fungus *P. chrysosporium* ATCC 34541 for the biological degradation of different batches of MIEX™ NOM concentrate and the effects of different characteristics of the NOM on the process and thus the resistance of the NOM to microbial removal were investigated. The effects of the C:N ratios in three different simple culture media inoculated with the fungus were studied. Three white-rot fungus and three yeast strains were tested to identify the most suitable organism for the bioremediation of the concentrated NOM from solution.

The effects of environmental conditions namely incubation temperature, carbon source level, type of inoculum and different initial NOM concentrations on the fungal growth and the production of the extracellular ligninolytic enzymes to enhance NOM removal by *Trametes versicolor* were determined. This was followed by the investigations of enhancement of enzyme production by studying the effects of supplements, temperature, pH and agitation. The enzymatic treatment of the concentrated NOM in vitro by *T. versicolor* was further examined to improve the NOM removal by determining the optimum temperature and pH. The biological treatment of NOM with the presence of two supplements was investigated to enhance ligninolytic enzyme induction, and hence improve NOM degradation.
Chapter 2  Literature Review

This chapter critically reviews literature relevant to the present research topic: the nature of natural organic matter (NOM), mechanisms of lignin degradation by white-rot fungi and applications of their enzymes, as well as a review of the potential applications of white-rot fungi for the removal of NOM from wastes arising from drinking water treatment.

2.1  Natural Organic Matter

2.1.1  Origin

Natural organic matter (NOM) varies in structure, binding and state of solubility in the soil and aqueous phase of the terrestrial and aquatic environments (Table 2.1) (Kördel et al. 1997). The total carbon contents of humic substances in soils (soil organic matter) and marine environments (marine humus) are $2500 \times 10^{12}$ kg and $3000 \times 10^{12}$ kg, respectively (Kördel et al. 1997).

Table 2.1  The various forms of NOM in different environments.

<table>
<thead>
<tr>
<th>Terrestrial Environment</th>
<th>Aquatic Environment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid phase of soils</td>
<td>Surface water</td>
</tr>
<tr>
<td>Soil water</td>
<td>• Freshwater</td>
</tr>
<tr>
<td>• Interstitial water</td>
<td>• Sea water</td>
</tr>
<tr>
<td>• Drain and seepage water</td>
<td>Sediment</td>
</tr>
<tr>
<td>Groundwater</td>
<td>• Solid phase</td>
</tr>
<tr>
<td></td>
<td>• Interstitial water</td>
</tr>
</tbody>
</table>

NOM, a complex mixture of organic carbon, can comprise as much as 90% of the total reduced carbon in aquatic ecosystems (Frazier et al. 2002). NOM can be categorised into dissolved (DOC) and particulate (POC) organic carbon. Aquatic humic substances contribute about 40-60% of DOC and comprise the largest fraction of NOM in waters (Kördel et al. 1997). The NOM load in ecosystems is formed from allochthonous and autochthonous sources (Frazier et al. 2002, Hood et al. 2003). Allochthonous DOC (derived from surface and subsurface leaching of vegetation and soils in the surrounding catchment) is typically enriched in fulvic acids and is highly aromatic and coloured, while autochthonous DOC (derived from algal and bacterial biomass in aquatic systems) is characterised by a lower fulvic acid content and C:N ratio (McKnight et al. 1994, Hood et al. 2003).
2.1.2 Composition and chemical structure

NOM mainly comprises carbon, oxygen, hydrogen and nitrogen. Table 2.2 shows the elemental compositions of some examples of different organic matter (Weber 2001).

Table 2.2 Elemental compositions of some examples of different organic matter (Weber 2001).

<table>
<thead>
<tr>
<th>Substances</th>
<th>% Dry ash-free basis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>Fulvic acids</td>
<td>44-49</td>
</tr>
<tr>
<td>Humic acids</td>
<td>52-62</td>
</tr>
<tr>
<td>Proteins</td>
<td>50-55</td>
</tr>
<tr>
<td>Lignin</td>
<td>62-69</td>
</tr>
</tbody>
</table>

Most NOM is comprised of a range of hydrocarbon compounds, from small hydrophilic acids, proteins and amino acids to larger humic and fulvic acids. The organic components found in NOM can range from largely aliphatic to highly aromatic (coloured), from highly charged to uncharged, and from apparent molecular weights around 10,000 down to 200 Dalton. Most characterisation studies reported that NOM in surface water, on average, showed a significant charge due to carboxylic acid groups, and some aromatic/hydrophobic character (Croué et al. 2000).

Figure 2.1 shows a general hydrocarbon structure of NOM that displays the diversity of moieties which appear in NOM (Shevchenko & Bailey 1996). It is not surprising that NOM is a complex mixture and structure of organic compounds with a great range of attached functional groups (amide, carboxyl, hydroxyl, ketone and various minor functional groups), given the wide variety of NOM sources, seasonal variations, and the numerous degradation and transformation mechanisms which affect NOM (Leenheer & Croué 2003).
Figure 2.1 General structure of NOM (Shevchenko & Bailey 1996).

NOM consists of non-humic (hydrophilic) and humic (hydrophobic) substances (Hood et al. 2003). The non-humic hydrophilic fractions are composed predominantly of well-defined chemical structures such as hydrophilic organic acids and low molecular weight compounds (carbohydrates, carboxylic acids, amino acids, lipids, proteins etc), which are easily attacked by micro-organisms (Motheo & Pinhedo 2000).

On the other hand, humic substances, which originate from microbial or chemical conversion of bacteria, plants and other living organism residues, are naturally occurring heterogeneous organic substances that are based on N-containing polymers. Humic substances comprise both aliphatic and aromatic high molecular weight components (Liao et al. 1982). They have a complex chemical structure with no defined chemical and physical properties, which are generally refractory to attack by micro-organisms (Motheo & Pinhedo 2000). Furthermore, they are categorised into three classes based on their solubility characteristics: humin, fulvic acid and humic acid (Weber 2001) (Figure 2.2).
Figure 2.2 Classification of NOM (http://www.humintech.com).

(i) Non-humic substances
Aiken et al. (1992) compared the characteristics of hydrophobic and hydrophilic acids isolated from different environments. The hydrophilic organic acids had lower carbon and hydrogen contents, higher oxygen and nitrogen contents, and were lower in molecular weight than hydrophobic organic acids. Moreover, the hydrophilic acids had a lower concentration of aromatic carbon and greater hetero-atom, ketone and carboxyl content than the fulvic acid (Aiken et al. 1992).

Other common types of non-humic substances found are carbohydrates. Carbohydrates constitute about 5-25% of organic matter in soils. They are divided into three subclasses: monosaccharides, oligosaccharides and polysaccharides, which are aldehyde and ketone derivatives of the higher polyhydric alcohols (Weber 2001).

(ii) Humic substances
Humic acid is not soluble in water under acidic conditions (pH< 2). Humic acids are the major extractable component of soil humic substances and are dark brown to black in colour. The fraction called fulvic acid is soluble in water under all pH conditions and remains in solution after removal of humic acid by acidification. Fulvic acid is light yellow to yellow-
brown in colour. Humins are soluble neither in bases nor in acids and are black in colour (Kördel et al. 1997, Weber 2001).

Figure 2.3 Chemical properties of humic substances (http://www.humintech.com).

These three fractions vary considerably in colour, C-, O- and N-contents. The differences between humic acid and fulvic acid can be described by disparity in molecular weight, numbers of functional groups (carboxyl, phenolic OH) and extent of polymerisation. The molecular weight distributions for aquatic fulvic acid and humic acid were reported to be between 500 and 2000 Dalton and between 2000 and 5000 Dalton, respectively (Howe & Clark 2002). The postulated relationships are represented in Figure 2.3, in which colour intensity, degree of polymerisation, carbon and oxygen contents, and acidity vary with increasing molecular weight. The high molecular weight humic acids have lower oxygen but higher carbon contents than the low molecular weight fulvic acids. Fulvic acids, which contain more functional groups of an acidic nature, especially COOH, have significantly higher acidities than humic acids (900-1400 meq/100 g cf. 400-870 meq/100 g) (Kördel et al. 1997, Weber 2001).

Humic acids are complex aromatic macromolecules with amino acids, amino sugars, peptides and aliphatic compounds involved in linkages between the aromatic groups. The structure of humic acid, shown in Figure 2.4, contains quinone structures, phenolic OH groups, nitrogen and oxygen as bridge units and COOH groups diversely located on aromatic rings (Weber 2001).
Another study characterised the general structure of aquatic humic acids and reported that they may consist of (a) single aromatics rings with mainly three to six substituents as alkyl side chain, carboxylic acid, ketone or hydroxyl groups, (b) short aliphatic carbon chains, and (c) polycyclic ring structures including polynuclear aromatics, polycyclic aromatic-aliphatic and fused rings involving furan and possibly pyridine (Liao et al. 1982).

![Figure 2.4 Model structure of humic acid](http://www.humintech.com)

Fulvic acids are identified as a heterogeneous mixture of medium molecular weight (400-2000 Dalton) yellow organic acids (McKnight et al. 1994). They are the most hydrophilic of humic substances, contain a wide variety of aromatic and aliphatic structures, and both are extensively substituted with oxygen-containing functional groups, particularly –COOH, –OH and C=O (Kördel et al. 1997, Weber 2001) (Figure 2.5).

![Figure 2.5 Model structure of fulvic acid](http://www.humintech.com)
2.2 Characterisation of NOM

Characterisation of NOM can be divided into two categories: studies of (a) whole water, where DOM is characterised in water and its inorganic constituents, and (b) DOM fractions isolated from water and inorganic constituents (Leenheer & Croué 2003).

2.2.1 Whole water characterisations

(i) TOC, DOC and BOM analyses

Total organic carbon (TOC) is the common measurement used to represent the NOM concentration in aquatic systems. Particulate organic matter (POC) is the organic carbon that is retained on a 0.45-μm-porosity membrane, while dissolved organic matter (DOC) is the fraction of the TOC smaller than 0.45 μm in diameter (Leenheer & Croué 2003).

According to Leenheer and Croué (2003), most NOM is considered to be refractory to rapid biodegradation but autochthonous NOM is more biodegradable than allochthonous NOM. Biodegradable organic matter (BOM) can be measured based on standard protocols such as biodegradation over a given time and is expressed as biodegradable dissolved organic carbon (BDOC) or assimilable organic carbon (AOC).

(ii) Spectrophotometric analysis

Absorbance at 446 nm (A446) is generally used as a measurement for colour whereas absorbance at 254 nm (A254) is an indicator of the presence of UV-absorbing components in NOM. Leenheer and Croué (2003) reported that the aromatic chromophores present in NOM molecules (particularly humic substances) absorb both visible and UV light.

DOC is a general indicator of the NOM content in a water sample however it does not give specific information on the potential for NOM to serve as disinfection by-product (DBP) precursors. Specific UV absorbance (SUVA), the ratio of A254 to DOC, is used to represent the enrichment of DOC in DBP precursors and as a measure of the aromaticity of the DOC (White et al. 2003). High SUVA waters suggest that the samples would likely result in DBP formation and are generally enriched in hydrophobic NOM, such as humic substances (Leenheer & Croué 2003). In addition to absorbance, fluorescence is also used to characterise NOM. Humic-type molecules are considered to be largely responsible for the fluorescence observed in natural waters (Leenheer & Croué 2003).
(iii) Size characterisation of NOM

Sequential ultrafiltration fractionation is used for low-resolution separations while size exclusion chromatography (SEC) is employed for higher-resolution size separations (Leenheer & Croué 2003). However, neither sequential ultrafiltration fractionation nor SEC gave absolute measures of the molecular weight of NOM (Schäfer et al. 2002).

Chin et al. (1994) suggested that number- ($M_n$) and weight-averaged ($M_w$) molecular weights for the humic substances could be determined by employing high performance size exclusion chromatography (HPSEC). This technique requires relatively small sample volumes and can be used with many samples without pre-concentration, thus allowing determinations of both molecular size and weight on whole-water samples. It also gives significant understanding of the nature of chemical interactions at the molecular level between dissolved organic carbon and other organic constituents (Chin et al. 1994).

### 2.2.2 Fractionation

DOM is generally characterised by isolating NOM into distinct fractions using resin sorbents (Leenheer & Croué 2003). The analytical method of DOC fractionation separates NOM into humic (hydrophobic acids, bases and neutrals) and non-humic (hydrophilic acids, bases and neutrals) substances based upon their adsorption on non-ionic and ion-exchange resin adsorbents (Leenheer 1981, Thurman & Malcolm 1981).

Chow et al. (2004) further developed a rapid fractionation system by employing DAX-8, XAD-4 and IRA-958 resins to obtain very hydrophobic acids (VHA), slightly hydrophobic acids (SHA), hydrophilic charged (CHA) and hydrophilic neutral (NEU) compounds. Table 2.3 provides a list of organic compounds classified according to different NOM fractions (Barber et al. 2001, Swietlik et al. 2004).
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Organic compound class</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humic substances or hydrophobic fractions (VHA + SHA)</td>
<td>Soil fulvic acids; C$_5$-C$_9$ aliphatic carboxylic acids; 1- and 2-ring aromatic carboxylic acids; 1- and 2-ring phenols; linear alkyl benzene sulphonate (LAS); LAS degradation products</td>
<td>Leenheer (1981)</td>
</tr>
<tr>
<td>Hydrophobic acid</td>
<td></td>
<td>Aiken et al. (1992)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Marhaba et al. (2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Barber et al. (2001)</td>
</tr>
<tr>
<td>Hydrophobic base</td>
<td>Portion of humic substances retained by XAD-8 resin at pH 7 which can be eluted by HCl; 1- and 2-ring aromatics amines except pyridine, proteinaceous substances, cationic surfactants</td>
<td>Leenheer (1981)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Marhaba et al. (2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Barber et al. (2001)</td>
</tr>
<tr>
<td>Hydrophobic neutral</td>
<td>Hydrocarbons; &gt;C$_5$ aliphatic alcohols, amides, esters, ketones, aldehydes; long chain (&gt;C$_9$) aliphatic carboxylic acids and amines; &gt;3-ring aromatic carboxylic acids and amines; chlorophyll and related pigments; LAS and optical brighteners</td>
<td>Leenheer (1981)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Marhaba et al. (2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Barber et al. (2001)</td>
</tr>
<tr>
<td>Non-humic substances or hydrophilic fractions (CHA + NEU)</td>
<td>&lt;C$_5$ aliphatic carboxylic acids; polyfunctional carboxylic acids; mixture of various hydroxy acids; LAS degradation products</td>
<td>Leenheer (1981)</td>
</tr>
<tr>
<td>Hydrophilic acid</td>
<td></td>
<td>Aiken et al. (1992)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Marhaba et al. (2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Barber et al. (2001)</td>
</tr>
<tr>
<td>Hydrophilic base</td>
<td>Amphoteric proteinaceous materials containing aliphatic amino acids, amino sugars, peptides and proteins; &lt;C$_9$ aliphatic amines; pyridine</td>
<td>Leenheer (1981)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Marhaba et al. (2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Barber et al. (2001)</td>
</tr>
<tr>
<td>Hydrophilic neutral</td>
<td>&lt;C$_5$ aliphatic amides, alcohols, aldehydes, esters, ketones; polyfunctional alcohols; carbohydrates; cyclic amides; polysaccharides</td>
<td>Leenheer (1981)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Marhaba et al. (2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Barber et al. (2001)</td>
</tr>
</tbody>
</table>
2.3 Impact of NOM on Water Quality and Treatment

A few decades ago research into the nature of NOM in potable water and methods for its removal was predominantly focused on removing colour and turbidity from public water supplies. Since then, several other problems associated with NOM have arisen in the treatment and delivery of drinking water. These include its demand for coagulants and disinfectants, its tendency to foul membranes and to interfere with the removal of other contaminants, as well as its potential to transport metals and hydrophobic organic chemicals and thus causing pipe corrosion in distribution systems (Jacangelo et al. 1995).

The presence of NOM in water is known to have adverse impacts on water treatment processes including coagulation, membrane filtration, GAC and disinfection. NOM can reduce the effectiveness of flocculation, which in turn increases the demand for coagulants due to its reactions with coagulants. Many studies have suggested that NOM is the most important membrane foulant as some DOC can penetrate and clog microfiltration and ultrafiltration membrane pores and thus contribute to fouling (Carroll et al. 2000, Howe & Clark 2002). NOM can also compete with other contaminants such as algal toxins and pesticides for adsorption sites on activated carbon and consequently block GAC pores. The fractions of NOM that cannot be successfully removed by conventional drinking water treatment can react with disinfectants to form DBPs, especially trihalomethanes (THMs) and haloacetic acids (HAAs), some of which are potentially carcinogenic in addition to being mutagenic (Carraro et al. 2000, Aoustin et al. 2001). Furthermore, NOM can serve as an electron donor in metal complexation, sorption of xenobiotics and adsorption on to mineral phases and on to activated carbon (Frimmel 1998).

Another concern of water utilities is the contribution of NOM as a substrate for bacterial regrowth (i.e., formation of biofilms) in drinking water distribution systems. This can lead to undesirable tastes, odour, particles, as well as the increased risk of gastrointestinal illness for drinking water consumers (Prévost et al. 1998). As a result of the deleterious effects of NOM on water quality, removal of NOM from drinking water is critical before delivery to the consumers. However, application of some treatments results in the generation of concentrated NOM wastes, such as sludges from alum precipitation, membrane treatment plants and MIEX regeneration, which must be treated prior to disposal.
2.4 White-rot Fungi

White-rot fungi belong to the wood-destroying basidiomycetes and are best known as the only micro-organisms responsible for the mineralisation of all major wood polymers, including lignin, cellulose and hemicellulose (Crawford & Crawford 1980). Several species have been investigated for their lignin-degrading capability, such as *Phanerochaete chrysosporium*, *Coriolus versicolor* (now called *Trametes versicolor*), *Pleurotus ostreatus*, *Phlebia radiata*, *Ceriporiopsis subvermispora*, *Panus tigrinus* and *Bjerkandera adusta*. The mechanisms for their wood biodegradation ability are dependent on the fungal species and conditions, e.g., *T. versicolor* degrades wood by simultaneous attack on both lignin and polysaccharides while *C. subvermispora* preferentially degrades lignin (Del Pilar Castillo 1997).

Research on the lignin-degrading enzyme system led to the detection of extracellular phenoloxidase enzymes in *P. chrysosporium* (Glenn *et al.* 1983, Tien & Kirk 1983, Kuwahara *et al.* 1984). The ligninolytic system of white-rot fungi is considered to contain a pool of enzymes, particularly lignin peroxidase (LiP), manganese-dependent peroxidase (MnP) and laccase (Lac), which are highly effective in oxidising and cleaving wood and lignin (natural components of the ecosystem), as well as various intractable xenobiotic pollutants structurally similar to lignin. Applications of the system present many potential advantages, such as the organisms do not have to adapt to the organic pollutants since the lignin-degrading system is triggered by the absence of a single nutrient, and the extracellular oxidative enzymes are not dependent upon the concentration of the pollutants (Del Pilar Castillo 1997); and the system is not specific and so allows the mineralisation of a broad range of persistent organic pollutants as opposed to bacterial systems which may require separate enzymes to catalyse the breakage of each bond type (Lonergan 1992). Moreover, the system is also recognised as a safe, versatile and economic biological treatment which is able to bio-transform hazardous compounds including xenobiotics; reduce ammonia, iron and manganese levels; as well as bio-oxidise assimilable organic carbon (AOC) that allows production of biologically stable water (Carraro *et al.* 2000).

White-rot fungi apply oxidative mechanisms for pollutant degradation and the organo-pollutants are metabolised through cycles of oxidation and subsequent quinone reduction reactions, leading to intermediates which undergo aromatic ring cleavage (Gold & Alic 1993). These allow the white-rot fungi to oxidise a wide range of recalcitrant organic compounds, for instance polyaromatic hydrocarbons (PAHs) (Muncnerova & Augustin 1994), polycyclic...
aromatic hydrocarbons (Bogan & Lamar 1996), polychlorinated biphenyls (Xu 1996), polychlorinated dibenzo(p)dioxins, and the pesticides DDT and lindane (Fujita et al. 2002). It has also been reported that *P. chrysosporium* ATCC 24725 is able to mineralise di-, tri-, tetra- and penta-chlorophenol (PCP) (Choi et al. 2002, Shim & Kawamoto 2002).

The control of culture parameters is important for optimum ligninolytic activity. Understanding the physiological bases of culture requirements will facilitate investigation of the extracellular ligninolytic enzyme system. It has been reported that factors such as temperature, pH, supplements (veratryl alcohol (VA), Tween 80 and lignocellulose residues), as well as agitation conditions affect the secretion of enzymes and thus the performance of white-rot fungi.

2.4.1 Effect of temperature and pH

Many researchers have investigated the effect of both temperature and pH on white-rot fungi for the bioremediation of different compounds. The effect of both factors varied when different substrates were investigated. It was reported that the optimum temperature and pH for an unidentified basidiomycete wood-rotting fungus used for the decolourisation of cotton bleaching effluent was found to be 27°C and pH 4-5, respectively (Zhang et al. 1999). Another study stated that the optimum pH for textile dyestuff decolourisation by *C. versicolor* MUCL was pH 4.5 and the fungus was capable of decolourising the dyestuff with lower efficiencies at pH 6 and 7 (Kapdan et al. 2000). Schliephake et al. (2000) determined the optimum temperature for a purified laccase from *Pycnoporus cinnabarinus* CBS 101046 during the degradation of the diazo dye Chicago Sky Blue. They established that the laccase was stable at 60°C for one hour and remained active in bioreactors at 37°C for 25 days (Schliephake et al. 2000).

The effect of temperature on textile dye decolourisation by *Trametes modesta*, investigated by Nyanhongo *et al.* (2002), showed that different dyes were decolourised at different rates at different temperatures. They reported that the rate of dye decolourisation due to laccase increased with temperature up to 50-60°C, after which it decreased (Nyanhongo *et al.* 2002). A catechol polymerisation study catalysed by laccase from *T. versicolor* ATCC 200801 demonstrated that the optimum pH for the oxidative process was pH 5 and the reaction rate increased exponentially with temperature up to 45°C, after which the rate tended to plateau (Aktas & Tanyolac 2003). Rancaño *et al.* (2003) reported that pH 5 was optimal for the
decolourisation of Phenol Red by laccase from *T. versicolor* CBS 100.29.

Dodor *et al.* (2004) worked with laccase from *T. versicolor* and observed that the rate of oxidation of anthracene and benzo[α]pyrene increased with increasing temperature up to 40°C, after which the rate started to decrease. Another group of researchers found that the optimum temperature and pH for the enzymatic decolourisation of Remazol Brilliant Blue R (RBBR) by *Funalia trogii* ATCC 200800 growing in a solid-state fermentation (SSF) medium containing wheat bran and soybean waste were 50°C and pH 3.0, respectively (Deveci *et al.* 2004).

### 2.4.2 Effect of supplements

There have been many investigations of the potential of different supplements (veratryl alcohol (VA), Tween 80 and lignocellulose residues) for inducing the production of the ligninolytic enzymes and thus improving the performance of white-rot fungi. It was reported that VA (3, 4-dimethoxybenzyl alcohol) is capable of stabilising LiP activity (Tsai 1991) and acts as a substrate for LiP (Feng *et al.* 1996). It is also believed that VA works as a redox mediator to facilitate the oxidation of chemicals (Harvey *et al.* 1986), has a protective effect against inactivation of certain LiP isoenzymes by H$_2$O$_2$ (Wariishi & Gold 1989), and has the ability to enhance the action of LiP on many substrates, including lignin (Hammel *et al.* 1993). The mediator concept is shown in Figure 2.6 for LiP catalysed oxidation of lignin.

![Figure 2.6 Veratryl alcohol as electron transfer mediator](Redrawn from Harvey *et al.* 1986).

Tween 80 (polyoxyethylene sorbitan monooleate), a non-ionic surfactant, is able to transform the cell membrane structure and promote permeation of LiP from the cell into the medium (Asther *et al.* 1987). Tween 80 can also protect LiP in culture fluids against mechanical
inactivation due to agitation (Venkatadri & Irvine 1990). They determined that the presence of Tween 80 caused a 1.3- to 1.4-fold increase in LiP enzyme activity, even under stationary conditions. Asther and co-workers (1987) explained that the saturated and unsaturated fatty acids, released by the hydrolysis of Tween 80 by most fungi, are involved in the activation of LiP production, either by providing an extracellular energy source for secondary metabolism or by serving as an inducer. However, the addition of Tween 80 as a culture supplement caused significant foaming due to aeration. The foaming caused by aeration is still of concern although Tween 80 has a protective effect against mechanical inactivation of the enzyme (Shim & Kawamoto 2002).

Recently, many studies have demonstrated the importance of utilising lignocellulose residues for enhancing the production of the extracellular phenoloxidase enzymes by white-rot fungi (Couto et al. 2001, Lorenzo et al. 2002, Couto et al. 2004, Kapich et al. 2004, Couto & Sanromán 2005). Lignocellulose residues are mainly composed of polysaccharides (cellulose and hemicellulose) and lignin and occur in a wide range of wastes from the agricultural and forestry industries. Lorenzo et al. (2002) showed that due to their cellulose content barley bran, grape stalks and grape seeds have significant potential to improve laccase production in submerged cultures of T. versicolor CBS 100.29. They investigated the potential enzymatic decolourisation of Phenol Red by the ligninolytic extracellular fluids obtained in the cultures with the aforementioned supplements and established that the cultures with barley bran and grape stalks had high decolourisation ability. The presence of the lignocellulose wastes provides the fungus an environment similar to its natural habitat (wood), and so may assist the stimulation of the secretion of the lignin-degrading enzymes.

Kapich et al. (2004) illustrated that the addition of solid lignocellulose-containing substrates such as hemp woody core and wheat straw to liquid medium led to the production of LiP and MnP in submerged cultures of P. chrysosporium ME-446. They suggested that the immobilisation of the mycelium on the surface of the substrates possibly provides a greater surface area and increases mass transfer and so improves the production of the enzymes. Moreover, the fungal mycelium may penetrate the lignocellulose substrate releasing additional water-soluble aromatic/phenolic substances, which in turn may increase the secretion of the ligninolytic enzymes (Kapich et al. 2004). In their review Couto and Sanroman (2005) noted that the use of organic wastes rich in lignin was ideal for the production of LiP, while utilisation of organic wastes rich in cellulose induced laccase production.
Table 2.4 shows the production of ligninolytic enzymes by different micro-organisms using SSF (Couto & Sanromán 2005).

**Table 2.4 Production of ligninolytic enzymes by SSF** (Couto & Sanromán 2005).

<table>
<thead>
<tr>
<th>Support</th>
<th>Micro-organisms</th>
<th>Ligninolytic enzymes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bagasse</td>
<td><em>Polyporus BH1, Polyporus BW1</em></td>
<td>LiP</td>
<td>Nigam <em>et al.</em> (1987)</td>
</tr>
<tr>
<td>Grape</td>
<td><em>C. versicolor, Panus tigrinus, P. chrysosporium</em></td>
<td>LiP</td>
<td>Golovleva <em>et al.</em> (1987)</td>
</tr>
<tr>
<td>wastewater</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat bran</td>
<td><em>P. pulmonarius</em></td>
<td>Lac</td>
<td>De Souza <em>et al.</em> (2002)</td>
</tr>
<tr>
<td>Wheat straw</td>
<td><em>C. versicolor, P. tigrinus, P. chrysosporium</em></td>
<td>LiP</td>
<td>Golovleva <em>et al.</em> (1987)</td>
</tr>
<tr>
<td></td>
<td><em>Pleurotus sp.</em></td>
<td>MnP, Lac</td>
<td>Lang <em>et al.</em> (1996)</td>
</tr>
<tr>
<td></td>
<td><em>P. chrysosporium</em></td>
<td>LiP, MnP</td>
<td>Castillo <em>et al.</em> (1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fujian <em>et al.</em> (2001)</td>
</tr>
<tr>
<td></td>
<td><em>P. ostreatus</em></td>
<td>Lac</td>
<td>Baldrian and Gabriel (2002)</td>
</tr>
<tr>
<td></td>
<td><em>P. pulmonarius</em></td>
<td>Lac</td>
<td>De Souza <em>et al.</em> (2002)</td>
</tr>
<tr>
<td>Wood</td>
<td><em>Bjerkandera sp. strain BOS55</em></td>
<td>LiP, MnP</td>
<td>Mester <em>et al.</em> (1998)</td>
</tr>
</tbody>
</table>
2.4.3 Effect of agitation

Early research showed that culture agitation suppressed both LiP production and lignin degradation in *P. chrysosporium* cultures (Kirk *et al.* 1978, Faison & Kirk 1985). It has been reported that agitation had no apparent effect on LiP formation by *P. chrysosporium*, but caused inactivation of the secreted enzyme (Venkatadri & Irvine 1990).

However, other investigations illustrated that LiP can be protected in agitated (200 rpm) submerged cultures of *P. chrysosporium* by supplementing with Tween 80 (Jäger *et al.* 1985). Lignin degradation has been achieved under agitated conditions (136 rpm), as reported by Reid *et al.* (1985). Another group of researchers demonstrated that agitation was very effective in improving (more than doubling) the rate of Orange II decolourisation by mycelial pellets of an unidentified basidiomycete fungus. This may be due to the improved mass transfer of oxygen and substrates in agitated cultures (Knapp *et al.* 1997).

2.5 Mechanism of Enzymatic Degradation by White-rot Fungi

White-rot fungi play a significant role in the recycling of lignin, capable of completely mineralising lignin to CO$_2$ and H$_2$O due to their non-specific extracellular ligninolytic enzyme system. Degradation of the complex irregular aromatic structure of lignin polymer by white-rot fungi enables them to access cellulose and hemicellulose, which they then utilise as carbon and energy sources (Leatham 1986, Kirk & Farrell 1987).

A general scheme for lignin biodegradation, which involves the oxidative reactions catalysed by LiP, MnP and Lac, is shown in Figure 2.7. Kirk and Chang (1975) studied the chemical changes of fungally degraded lignin and showed that it had decreases in phenolic hydroxyl, aliphatic hydroxyl and methoxyl contents. Furthermore, higher contents of $\alpha$-carbonyl and conjugated carboxyl groups in the degraded lignin were obtained. Another observation was that the degraded lignin contained oxidised side chains and aliphatic residues resulting from oxidative cleavage of aromatic rings (Kirk & Chang 1975).

The lignin-degrading enzyme system of white-rot fungi has been widely studied. A number of white-rot fungi and their enzymes have been used successfully in various configurations in different types of industrial applications. Since a diverse range of recalcitrant organic pollutants contain a chemical structure similar to lignin, the ligninolytic system and oxidative mechanism is considered to be involved in the degradation of the pollutants (Gold & Alic
1993) as well as humic acids (Blondeau 1989), and so of naturally occurring organic matter due to the similarity between the structures of lignin and NOM.

![Diagram showing the biodegradation of lignin](image)

**Figure 2.7** Proposed schemes for the biodegradation of lignin (Redrawn from Leisola & Garcia 1989).

Brief descriptions of three major extracellular phenoloxidase enzymes secreted by white-rot fungi, which are involved in the oxidative degradation mechanism, are described below.

### 2.5.1 Lignin peroxidase

Lignin peroxidase (LiP) is a glycoprotein that contains one mole of iron protoporphyrin IX as a prosthetic group. It has a series of isoenzymes with molecular weight of 41,000-42,000 (Kirk & Farrell 1987, Gold et al. 1989). The enzyme can be assayed by the oxidation of veratryl alcohol (VA) to veratraldehyde at 310 nm (Tien & Kirk 1984). Fungi that were identified as producing LiP include *P. chrysosporium*, *T. versicolor*, *Pleurotus ostreatus*, *Phlebia tremellosus* etc.

LiP has a relatively high redox potential and has no substrate specificity. LiP has been shown to oxidise phenolic and non-phenolic lignin related compounds as well as a wide variety of model lignin and related compounds (Barr & Aust 1994). Among the oxidation reactions catalysed by LiP are the cleavage of the Cα-Cβ and aryl Cα bonds, ring cleavages in β-O-4 compounds, aromatic ring opening, demethylation and phenolic oxidation (ten Have &
Teunissen 2001). All of these reactions are involved in the same mechanism in which the oxidised enzyme intermediates LiP (I) and LiP (II) catalyse the initial one-electron oxidation of the substrate to yield an aryl cation radical, followed by a series of non-enzymatic reactions to yield the final products (Gold et al. 1989). This is illustrated in Figure 2.8 for the LiP-catalysed oxidation of a β-1 diarylpropane dimeric model compound, showing the mechanism of Cα-Cβ cleavage.

![Mechanism of Cα-Cβ cleavage by LiP](image)

**Figure 2.8**  Mechanism of Cα-Cβ cleavage by LiP (Redrawn from Gold et al. 1989).

The catalytic cycle of LiP is illustrated in Figure 2.9 (Gold et al. 1989). The native enzyme reacts with H2O2 forming the two-electron oxidised intermediate, LiP (I), which then oxidises the lignin substrate (RH) to produce the one-electron oxidised intermediate LiP (II) and a
substrate radical (R'). The LiP (II) is then reduced back to the resting enzyme state by oxidising a second substrate compound while the free radical can undergo a range of reactions. With excess H₂O₂ LiP (II) can be converted to an inactive form of the enzyme LiP (III) (Tien 1987, Gold et al. 1989, Del Pilar Castillo 1997). However, as mentioned in Section 2.4.2, the presence of VA can protect against inactivation of the LiP by the excess H₂O₂ as well as mediate the oxidation of lignin as shown in Figure 2.6.

![Catalytic cycle of lignin peroxidase](image)

**Figure 2.9** Catalytic cycle of lignin peroxidase (Redrawn from Gold et al. 1989).

### 2.5.2 Manganese-dependent peroxidase

The discovery of LiP initiated the search for other oxidative enzymes and led to the detection of manganese-dependent peroxidase (MnP). MnP is also a heme peroxidase and a glycoprotein. Like LiP they have a family of isoenzymes, containing one iron protoporphyrin IX group per mole of enzyme with molecular weight approximately 46,000 (Kuwahara et al. 1984). MnP has been shown to degrade a wide range of phenols and dyes (Kuwahara et al. 1984) by oxidising Mn (II) to the oxidant Mn (III) (Kirk & Farrell 1987), which diffuses from the active site of the enzyme and reacts with different phenolic substrates.
The catalytic cycle of MnP is illustrated in Figure 2.10 (Gold et al. 1989). It is basically the same as for LiP, except that Mn (II) is required to complete the cycle. As shown in Figure 2.10, the resting enzyme reacts with \( \text{H}_2\text{O}_2 \) to form MnP (I), which is then transformed to MnP (II) by oxidising one equivalent of Mn (II) to yield Mn (III). A second Mn (II) is then responsible to reduce MnP (II) back to the resting enzyme. Similarly, the MnP (I) can oxidise phenolic substrates (RH), but at a slower rate. Phenolic compounds are, however, not capable of efficiently converting MnP (II) back to the resting enzyme. This may be due to the \( \text{Fe}^{4+} = \text{O} \) centre in MnP (II) being partially buried and so the site is not available to organic substrates. Thus, Mn (II) is essential for the completion of the cycle (Wariishi et al. 1988, Del Pilar Castillo 1997).

![Catalytic cycle of manganese peroxidase](Redrawn from Gold et al. 1989).

The oxidation of a free phenolic \( \beta \)-1 lignin dimer by MnP is shown in Figure 2.11. The initial reaction involves a one-electron oxidation of the phenol to produce a phenoxy radical intermediate. As a result, dehydrogenation would yield the ketone (B), alkyl phenyl cleavage of the radical intermediate would form products (C) through (E) and finally, \( \text{C}_\alpha\text{-C}_\beta \) would yield the products (F) through (H) (Gold et al. 1989).
2.5.3 Laccase

Laccase (Lac) is an extracellular glycosylated enzyme and belongs to the copper-containing polyphenol oxidase family. The enzyme is generally larger than LiP and MnP, having a molecular weight around 60,000 (ten Have & Teunissen 2001). Laccase is capable of catalysing the oxidation of phenolic and non-phenolic compounds (Bourbonnais & Paice 1990) and is able to decolourise a wide range of synthetic dyes (Rodriguez et al. 1999, Swamy & Ramsay 1999). It was reported that fungal laccase worked synergistically with
MnP in oxidising phenols and phenolic substructures of lignin through decarboxylation and subsequent demethylation of methoxy groups (Galliano et al. 1991, Schlosser & Höfer 2002).

Kawai (1988) proposed that laccase not only catalyses alkyl-aryl cleavage and \( C_\alpha \) oxidation, but also catalyses \( C_\alpha-C_\beta \) cleavage of phenolic moieties via phenoxy radicals. The proposed pathway, which proceeds via the formation of oxygen mediated phenoxy radicals of the phenolic units by laccase, is illustrated in Figure 2.12.

**Figure 2.12** Proposed degradation of phenolic \( \beta \)-1 model compounds by laccase from *C. versicolor* (Kawai et al. 1988).
2.6 Potential Applications of White-rot Fungi in Bioremediation

As outlined in Chapter 1, there are several available processes for the removal of NOM in waste and drinking water treatment industries. However, all have advantages and disadvantages. Conventional water treatments have been identified as having low removal efficiency and high operating cost, are mostly based on chemical addition and applicable to a limited concentration range, as well as producing sludge and residuals (Vickers et al. 1995, Burton et al. 1999). The NOM-containing wastes generated from alum precipitation, membrane process plant and the anionic exchange MIEX™ process can be problematic regarding disposal. Conventional coagulation, which requires the addition of a coagulant such as alum, ferric salts or polyaluminium chlorides, has low NOM removal efficiency (10-50%) and generates sludge disposal problems (Jacangelo et al. 1995, Vickers et al. 1995). In addition, the control of coagulant addition and adjustment in pH are necessary, as these must be adjusted with any change in the raw water (Jacangelo et al. 1995, Vickers et al. 1995). Some studies on coagulation suggested that lower molecular weight, hydrophilic, uncharged and fulvic acid-like components still remain in natural waters after the treatment (Chow et al. 1999, Drikas et al. 2003, Chow et al. 2004). This is consistent with Page et al. (2003), who established that the proportion of polysaccharide-derived compounds (i.e., of hydrophilic character) generally increased after alum treatment, indicating that these compounds are refractory to alum coagulation.

Adsorption by granular or powdered activated carbon (GAC or PAC), which is widely used in the United States, has limitations since its adsorption capacity is limited and may be exhausted after a short period. Consequently, frequent reactivation or replacement of activated carbon is necessary (Jacangelo et al. 1995). Ozonation, which is effective in transformation of refractory NOM to biodegradable dissolved organic carbon (BDOC), has some limitations. These include high operating costs and the possibility of mutagen (aldehydes and other ozonation by-products, e.g. ketones, bromates) formation (Gilli et al. 1990, Kirisits et al. 2001). Furthermore, the maximum BDOC production by ozonation is only approximately 30% of the total DOC in raw water even if the ozone dose is increased, due to the fact that BDOC may also consume ozone (Volk et al. 1993, Wricke et al. 1996, Nishijima et al. 2003).

Membrane filtration has become an established process in water treatment industry. However, one of the most significant factors limiting the implementation of membrane filtration is fouling. Colloidal matter may cause fouling by forming a cake at the membrane surface, while
dissolved matter, some of which can penetrate pores, causes fouling by forming a surface cake, penetrating and clogging pores, or adsorbing within membrane pores to reduce the pore diameter (Carroll et al. 2000, Howe & Clark 2002). These limitations reduce the effectiveness, lead to frequent cleaning, and so reduce the lifetime of membranes. Furthermore, membrane filtration can generate sludge, and thus a sludge disposal problem.

Consequently, reduction of treatment costs and development of energy-efficient waste treatment processes are needed as much sludge, some of which contains NOM, is generated in NOM removal processes. Biological treatment can be an option for the breakdown of the NOM, as the treatment would overcome some problems associated with conventional treatment processes, including chemical usage and sludge management. Bioremediation technology is viewed as an attractive approach to the removal of NOM as it is ‘natural’ and a potentially chemical-free process, which should have good public acceptance, and leads to minimal waste production.

White-rot fungi are recognised for their ability to degrade lignin and an array of persistent aromatic pollutants due to their non-specific extracellular ligninolytic enzymes and their ability to adapt to severe environmental constraints (Lonergan 1992). Del Pilar Castillo (1997) suggested that since the lignin-degrading system of P. chrysosporium has been shown to remove pollutants such as pesticides, it could be an option for the treatment of contaminated farmland. The wastewater effluents of textile, paper, printing and dye industries are highly coloured and contain toxic aromatic amines (Kapdan et al. 2000). Kapden et al. (2000) investigated the effects of environmental conditions such as pH, carbon source and dyestuff concentration on the textile dyestuff decolourisation performances of C. versicolor MUCL. Complete decolourisation was observed for initial dyestuff concentrations lower than 500 mg/L whereas any concentrations beyond 1200 mg/L may have a toxic effect on the fungus. They established that biodegradation rather than adsorption of dyestuff on the fungus (<20%) was the major mechanism involved in the removal of dyestuff concentrations up to 1200 mg/L.

Due to the similarity of parts of the structure of NOM with lignin, several researchers have shown the capability of a number of white-rot fungi to decolourise and degrade humic substances, among which were P. chrysosporium (Blondeau 1989, Ralph & Catcheside 1994), Trametes (Coriolus) species (Dehorter & Blondeau 1992, Yanagi et al. 2003), Clitocybula dusenii (Ziegenhagen & Hofrichter 1998) and Panus tigrinus (Zavarzina et al. 2004).
Blondeau (1989) reported that the mineralisation of humic acids by LiP of *P. chrysosporium* BKM-F 1767 occurred during secondary metabolism in nitrogen-limited medium, however, low activity of LiP was produced in the presence of humic acids. A reduction in the high molecular weight range and no accumulation of low molecular weight species were obtained after the fungal treatment. Ralph and Catcheside (1994) studied the decolourisation and depolymerisation of low rank coal (lignin-like polymers) with *P. chrysosporium* and established that the degradation of the coal coincided with the presence of extracellular LiP and MnP. They also suggested that aryl cation radicals and possibly Mn$^{3+}$ generated by LiP and MnP, respectively, diffused into the coal matrix and initiated the free radical oxidation reactions resulting in the fission of carbon bonds and thus depolymerisation, as has been proposed for lignin mineralisation.

Dehorter and Blondeau (1992) illustrated a relationship between the degradation of humic acid and extracellular enzyme activity for *P. chrysosporium* and *T. versicolor*, showing increased formation of extracellular LiP and MnP with increasing humic acid concentration, which was in contrast to the results of Blondeau (1989). They found that *T. versicolor* was more effective in degrading humic acids than *P. chrysosporium* with MnP as a key enzyme responsible for mineralisation. A study by Yanagi *et al.* (2003) with *Coriolus consors* IFO 9078 demonstrated that decolourisation of humic acids with different chemical properties ranged from 9 to 40%, with higher aromaticity and humification providing higher resistance to microbial decolourisation.

*C. dusenii* b11 was another white-rot fungus investigated for its ability to degrade humic acids obtained from low rank coal. Ziegenhagen and Hofrichter (1998) determined the optimum conditions for the action of MnP and established that the MnP-catalysed depolymerisation of humic acids *in vitro* produced low molecular weight fulvic acids by breaking down covalent bonds. Recently Zavarzina *et al.* (2004) studied the changes in the structures of soil- and peat-derived humic acids after biotransformation by laccase from the white-rot fungus *P. tigrinus* 8/18. It was reported that the purified laccase was able to polymerise and depolymerise humic acids, and the transformations were dependent upon the nature and properties of humic acids.

Melanoidin, a highly coloured constituent of the spent wash largely produced in the ethanol industry, has high environmental pollution potential (Fahy *et al.* 1997, Dahiya *et al.* 2001). The brown melanoidin polymers, formed by the Maillard amino-carbonyl reaction (Wedzicha & Kaputo 1992), have antioxidant properties and structures similar to wood and NOM. Fahy
at al. (1997) and Dahiya et al. (2001) studied a microbial decolourisation process for melanoidin pigments present in spent wash using *P. chrysosporium*. Dahiya et al. (2001) reported that 80% colour removal was achieved and showed that the rate of melanoidin decolourisation for the high molecular weight fractions was faster than for the low molecular weight fractions.

Rojek et al. (2004) have shown that *Phanerochaete chrysosporium* ATCC 34541 was able to remove 40-50% NOM from solution, although this was shown to be mainly due to adsorption and to be partially metabolically linked. Following the work of Rojek (2003), further investigations were conducted to examine the bioremediation of NOM wastes to develop an improved biological system applicable to wastewater treatment.
Chapter 3  Materials and Methods

3.1  NOM Samples

The highly coloured MIEX™ NOM concentrate from Hope Valley Reservoir, located in South Australia, was utilised as a source of organic matter throughout the experiments. The NOM concentrate was obtained from the regeneration process of the strong base magnetic ion exchange (MIEX) resin, a recently developed process for the removal of dissolved organic carbon (DOC) (Slunjski et al. 2000).

The concentrate was filtered (0.45 μm hydrophilic PVDF, Millipore Millex-HV) and stored at 4°C prior to treatment and analysis. The characteristics of the NOM concentrates used in this study, which were collected at different times, are tabulated in Table 3.1.

Table 3.1  Characterisation of MIEX™ NOM concentrates.

<table>
<thead>
<tr>
<th>Description</th>
<th>Units</th>
<th>MIEX™ NOM Concentrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch</td>
<td>NOM 1</td>
<td>NOM 2</td>
</tr>
<tr>
<td>Collection Date</td>
<td>March, 2001</td>
<td>July, 2003</td>
</tr>
<tr>
<td>pH</td>
<td>7.63</td>
<td>7.15</td>
</tr>
<tr>
<td>Absorbance at 446 nm (1:100)(^a)</td>
<td>cm(^{-1})</td>
<td>0.079</td>
</tr>
<tr>
<td>Absorbance at 254 nm (1:2000)(^b)</td>
<td>cm(^{-1})</td>
<td>0.114</td>
</tr>
<tr>
<td>DOC</td>
<td>g C/L</td>
<td>6.5</td>
</tr>
<tr>
<td>SUVA(^c)</td>
<td>L.mg(^{-1}).m(^{-1})</td>
<td>3.5</td>
</tr>
</tbody>
</table>

\(^a\) Dilution factor of 100
\(^b\) Dilution factor of 2000
\(^c\) SUVA (Specific UV absorbance) = \( \frac{A_{254}}{DOC} \)
3.2 Micro-organisms

*Phanerochaete chrysosporium* strains ATCC 34541 and ATCC 24725, *Trametes versicolor* strain ATCC 7731 and *Saccharomyces* species arbitrarily denoted 1, 2 and 3 (isolated from NOM concentrate in the RMIT University laboratory) were used in this study. *P. chrysosporium* and the yeast strains were maintained by subculturing monthly on Waksman medium agar slants for 3-4 days whereas *T. versicolor* was grown on 2% (w/v) malt extract agar (MEA) for 4-5 days. All organisms were incubated at 30°C and then maintained at 4°C prior to use.

The composition of the Waksman medium agar slants for fungal maintenance is detailed in Table 3.2. All chemicals were of AR purity.

<table>
<thead>
<tr>
<th>Table 3.2 Composition of Waksman medium agar slants.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium component</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>Agar</td>
</tr>
<tr>
<td>Mycological peptone</td>
</tr>
<tr>
<td>D-Glucose</td>
</tr>
<tr>
<td>NH₄Cl</td>
</tr>
<tr>
<td>KH₂PO₄</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
</tr>
</tbody>
</table>

3.3 Medium and Culture Conditions

Three simple growth media, i.e., modified Waksman medium (Booth 1971), Fahy medium (Fahy *et al.* 1997) and Fujita medium (Fujita *et al.* 2002) were employed. Medium was first adjusted to pH 4.5 before being sterilised by autoclaving at 121°C for 20 minutes. The composition of the listed media is given in Table 3.3.

Medium was prepared and 200 mL added to 500 mL Erlenmeyer flasks, which were then autoclaved. The shake flasks were then supplemented with filter sterilised (0.45 μm hydrophilic PVDF, Millipore Millex-HV) NOM (final concentrations of 100-700 mg C/L) and inoculated with the fungal or yeast strains (Section 3.4). The cultures were incubated at 30°C or 36°C as indicated for various periods at 130 rpm.
Table 3.3 Composition of growth media.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Glucose (g/L)</th>
<th>NH₄Cl</th>
<th>NH₄NO₃</th>
<th>KH₂PO₄</th>
<th>MgSO₄&lt;br/&gt;4 (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waksman</td>
<td>2.0 or 5.0ᵃ</td>
<td>0.5</td>
<td>-</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Fahy</td>
<td>25.0</td>
<td>0.5</td>
<td>-</td>
<td>2.5</td>
<td>1.25</td>
</tr>
<tr>
<td>Fujita</td>
<td>10.0</td>
<td>-</td>
<td>0.1</td>
<td>1.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

ᵃ Glucose content varied according to the experiment.

3.4 Preparation of Inoculum

3.4.1 Fungal inoculum

The fungi were inoculated as either a spore suspension or as plugs, as indicated. Spore suspensions were prepared by washing agar plates with sterilised water and then filtering through sterile glass wool. Spore concentration was determined by measuring the absorbance at 650 nm and calculated on the basis that A₆₅₀ = 1.0 cm⁻¹ corresponds to 5.0 x 10⁶ spores/mL (Kirk et al. 1978). Spore suspension (10 mL) was then added to the culture media to attain a concentration of 1.0-1.5 x 10⁵ spores/mL. Inoculation as plugs involved the addition of three agar plugs of 1 cm² each excised from a fungal colony actively growing on a MEA plate with a sterilised cutter.

3.4.2 Yeast inoculum

Yeast cultures were prepared with a loopful of inoculum from malt extract agar (MEA) plate.

3.5 Supplements

In some experiments, as indicated, two supplements were employed: wheat bran and Tween 80. Wheat bran (4.5 g/L, Kellogg’s) and Tween 80 (0.5% v/v, Merck) were added to the growth medium prior to sterilisation.

3.6 Analytical Methods

3.6.1 pH

A HACH Sension 156 Multiparameter Meter was used to measure medium pH. Any adjustment of pH was made with 0.1 M, 0.5 M or 1.0 M NaOH and H₂SO₄, as appropriate.
3.6.2 **Dissolved organic carbon**

DOC concentration was determined using a total organic carbon (TOC) analyser (Sievers, Model 820). Samples were filtered (0.45 μm hydrophilic PVDF, Millipore Millex-HV) and diluted as required with Milli-Q water prior to analysis.

3.6.3 **Absorbance**

Absorbance measurements were performed with a double beam scanning UV/vis spectrophotometer (Unicam, Model UV2) fitted with a cell of 1 cm pathlength. The absorbance of NOM solution was measured at both 446 nm (colour) and 254 nm (UV-absorbing components). The correlations between NOM concentration for the three different preparations and absorbance at 446 nm and 254 nm are provided in Appendix 1. Samples were centrifuged until the solution was clear and were diluted to 1:10 with Milli-Q water prior to $A_{254}$ measurements.

3.6.4 **Determination of absorbance correction factor**

The absorbance of NOM varies with pH, therefore corrections were applied where necessary, using the absorbance at initial pH as reference. NOM in medium at varying concentrations (50, 100 and 200 mg C/L) and pH (2-6) for the three different NOM preparations were prepared in duplicate and the absorbances at 446 nm and 254 nm were measured. As the pH of the culture fluid was usually in the range 2-6, the change in absorbance (%) was calculated using the absorbance at pH 6 as initial value, and pH 2 and its corresponding absorbance as final value. An almost linear relationship between pH versus absorbance was obtained hence the least squares method was applied to calculate the formula for the relationship between pH change (difference from initial to final) and the absorbance correction factor (%). Thus the formula for calculating percent change in absorbance for all NOM concentrations for any change in pH was established.

All data for 446 nm and 254 nm presented in this study have been corrected for pH. Plots illustrating the influence of pH on absorbances at 446 nm and 254 nm for the three NOM preparations and description of the calculation of corrected absorbance can be referred to in Appendix 2.
3.6.5 Determination of glucose concentration

Reducing sugar concentrations of samples were measured by the 3’, 5’-dinitrosalicylic acid (DNS) method (Miller 1959) using D-glucose as a standard. A typical standard curve for glucose determination is shown in Appendix 3.

3.6.6 Dry weight of biomass

Dry weight of biomass for both fungal and yeast strains was determined at the end of fermentations by filtering the biomass on pre-weighed dried membrane filters (0.45 μm Whatman WCN sterile membranes), washing with distilled water and then drying them in an oven at 90°C to constant weight. The dry weight of the biomass was then calculated by difference.

3.6.7 Enzyme assays

All enzyme assays were performed at 50°C, unless stated otherwise, as the reaction rates were slow and so would have been even slower at lower temperatures.

(i) Laccase activity

Laccase (Lac) activity was determined spectrophotometrically as described by Coll et al. (1993) with guaiacol as a substrate. The oxidation of guaiacol (Sigma, 100%) to the polymer tetraguaiacone was monitored by increase in absorbance at 465 nm. The activity was expressed in U/mL where one unit (U) of Lac activity was defined as the amount of the enzyme that caused an increase of one absorbance unit per minute (Coll et al. 1993). The reaction (Figure 3.1) was started by addition of guaiacol.

![Proposed mechanism for the oxidation of guaiacol by laccase](niwa2004.png)

Figure 3.1 Proposed mechanism for the oxidation of guaiacol by laccase (Niwa 2004).
The reaction mixture (3 mL) contained:

1.0 mM guaiacol 300 μL
0.2 M sodium acetate-acetic acid buffer, pH 4.5 1800 μL
Distilled water 600 μL
Extracellular culture fluid 300 μL

(ii) Lignin peroxidase

Lignin peroxidase (LiP) activity was measured according to Tien and Kirk (1988) by monitoring the oxidation of veratryl alcohol (VA) (Aldrich, 96%) to veratraldehyde at 310 nm. Enzyme activity was calculated from the molar extinction coefficient of \( \varepsilon = 9.30 \text{ mM}^{-1}\text{cm}^{-1} \) and expressed in units (U) which correspond to 1.0 μmole of veratraldehyde produced per minute (Tien & Kirk 1988). The reaction (Figure 3.2) was started by addition of hydrogen peroxide.

![Figure 3.2](image.png)

**Figure 3.2** Proposed mechanism for catalysing reduction of VA by LiP (Aust 1995).

The assay mixture (3 mL) included:

20.0 mM VA 300 μL
0.2 M sodium acetate-acetic acid buffer, pH 3.0 840 μL
4.0 mM \( \text{H}_2\text{O}_2 \) 300 μL
Extracellular culture fluid 1560 μL

(iii) Manganese-dependent peroxidase

Manganese-dependent peroxidase (MnP) activity was assayed according to Wariishi *et al.* (1992) by measuring the oxidation of 2, 6-dimethoxyphenol (DMP) (Aldrich, 99%) to coerulignone at 469 nm. The activity was expressed in units (U) and determined from the molar extinction coefficient of \( \varepsilon = 49.6 \text{ mM}^{-1}\text{cm}^{-1} \). One unit of MnP activity is defined as the
amount of the enzyme catalysing the formation of 1.0 \textmu mole of coerulignone per minute (Wariishi et al. 1992). The reaction is shown in Figure 3.3.

![Proposed mechanism for the oxidation of DMP by MnP](image)

**Figure 3.3 Proposed mechanism for the oxidation of DMP by MnP** (Wariishi et al. 1992).

The assay mixture (3 mL) contained:

- 0.5 mM DMP \(300 \mu\text{L}\)
- 0.2 M sodium acetate-acetic acid buffer, pH 4.5 \(1800 \mu\text{L}\)
- 1.0 mM MnSO\(_4\) \(300 \mu\text{L}\)
- 0.5 mM H\(_2\)O\(_2\) \(300 \mu\text{L}\)
- Extracellular culture fluid \(300 \mu\text{L}\)

### 3.6.8 High performance size exclusion chromatography

The molecular weight distribution of samples was determined using high performance size exclusion chromatography (HPSEC) at the Australian Water Quality Centre (AWQC), Adelaide.

The analysis was conducted using a Waters 2690 Alliance system with a temperature controlled oven at 30\(^{\circ}\)C and a Shodex KW 802.5 glycol functionalised silica gel column with a Waters 996 Photo Diode Array detector set at 260 nm. The column was calibrated with polystyrene sulphonate standards and the apparent molecular weight (Dalton) of NOM was calculated from the linear regression of the relationship between the retention time (t, minutes) and the logarithm of molecular weight of the standards (log (\(M_w\))): \[ \log(M_w) = -0.399 \times t + 7.205 \] (Appendix 4).

The weight average molecular weight (\(M_w\)) and the number average molecular weight (\(M_n\)) were calculated as per Equation 3.1 and Equation 3.2, respectively.
\[ M_w = \frac{\sum n_i M_i^2}{\sum n_i M_i} \]  \hspace{1cm} \text{Equation 3.1}

\[ M_n = \frac{\sum n_i M_i}{\sum n_i} \]  \hspace{1cm} \text{Equation 3.2}

where \( n_i \) is the number of molecules of weight \( M_i \). Polydispersity was calculated as the ratio \( M_w : M_n \).

3.6.9 Fractionation of NOM

A NOM fractionation system as designed by Chow et al. (2004) was constructed as shown in Figure 3.4. The system allows fractionation of the NOM into four categories: very hydrophobic acids (VHA), slightly hydrophobic acids (SHA), hydrophilic charged (CHA) and hydrophilic neutral (NEU) compounds. The VHA, SHA and CHA fractions were adsorbed by DAX-8 resin, XAD-4 resin and IRA-958 resin respectively; and the NEU fraction was the effluent from the IRA-958 column.

Three 20 cm glass columns for DAX-8, XAD-4 and IRA-958 resins respectively were set up in series as shown in Figure 3.4 after exhaustive cleaning of resins with methanol and Milli-Q water. Resin-water slurries were added to give bed volumes of approximately 14.7 mL, 14.1 mL and 20.6 mL respectively. Each bed was backwashed with 2-3 L Milli-Q water to classify the resin particles and to remove air bubbles and debris (Chow et al. 2004).

Before fractionation, samples were filtered through a 0.45 \( \mu \)m hydrophilic PVDF (Millipore Millex-HV) and acidified to pH 2.0 with concentrated HCl. The pH-adjusted samples were then passed through the DAX-8 column at the rate of 0.2 bed volumes/min. The first two bed volumes were discarded before collecting the effluent. A sub sample of 100 mL was stored for TOC, \( A_{446} \) and \( A_{254} \) assays. The remaining effluent was then passed through the second (XAD-4) column. The same procedures were followed except that the effluent from the XAD-4 column was adjusted to pH 8.0 with NaOH solution before pumping through the last (IRA-958) column. The DOC of each fraction was calculated by the difference between the DOC of effluents of the columns.
Figure 3.4  Schematic diagram of the fractionation unit (Chow et al. 2004).
Chapter 4  Decolourisation and Bioremediation of MIEX™ NOM

Following the work of Rojek (2003), preliminary fermentations using *P. chrysosporium* ATCC 34541 for the removal of NOM from various batches of MIEX NOM concentrate were conducted to identify the effect of different characteristics of NOM on the process, and so the applicability of the process to the treatment of NOM waste arising from drinking water.

Three NOM preparations (refer to Materials and Methods-Section 3.1 where described) were fractionated before the treatment to determine the natures of the organic compounds present. A comparison of the changes in colour ($A_{446}$), UV-absorbing components ($A_{254}$) and molecular weight distribution (HPSEC) was carried out after the fermentations to establish the fractions removed by *P. chrysosporium*. This was followed by the selection of a simple medium from three growth media with different C:N ratios. Three strains of white-rot fungi (*P. chrysosporium* ATCC 34541 and 24725, and *T. versicolor* ATCC 7731) and yeast (*Saccharomyces* species 1, 2 and 3) were investigated to develop an improved NOM waste biodegradation system.

### 4.1 Removal of Different Preparations of NOM by *P. chrysosporium* ATCC 34541

The three NOM preparations were added to 200 mL (final concentration of 100 mg C/L) of Waksman medium (2 g/L glucose), inoculated with 10 mL of spore suspension of *P. chrysosporium* ATCC 34541, and incubated at 36°C and 130 rpm for five days. The fermentations for each NOM preparation were performed in duplicate. Data points represent mean values of duplicates.

The trends of the history plots for the three NOM preparations were similar, and the plot for NOM 3 (Figure 4.1) is shown as it gave the highest reduction in $A_{446}$ and $A_{254}$. The pH dropped markedly over the first two days of incubation and then plateaued. The change in pH occurred concurrently with the decrease in glucose content. The drop in pH was probably due to the accumulation of organic acid as metabolite or by the freeing of hydrogen ions in substrate transfer by the fungus (Griffin 1994). This may support biosorption as the bioadsorptive capacity of a fungus increased with decreasing pH for humic acids as reported by Zhou and Banks (1991). The fungus consumed only approximately half of the glucose (1.0 g/L) provided over the incubation period. This is consistent with the results found by Rojek (2003), where the fungus consumed only 1-1.2 g/L glucose even though higher initial glucose concentrations (4 and 10 g/L) were supplied. $A_{446}$ decreased for the first three days and then
plateaued whereas $A_{254}$ decreased gradually during the whole fermentation. This is contrary to the findings of Blondeau (1989), where the decolourisation of humic acids by *P. chrysosporium* BKM-F 1767 only started after an initial lag phase of four days and continued up to day 15 and suggested that the lignin-degrading system played a role in the humic acid decolourisation.

![Graph](image)

**Figure 4.1** History plot showing pH, glucose consumption, $A_{446}$ and $A_{254}$ for NOM 3 incubated with *P. chrysosporium* ATCC 34541 at 36°C and 130 rpm for five days. ($A_{254}$ represents readings of 1/10 dilution of culture medium)

Figure 4.2 clearly indicates that there was a decrease in absorbance at 446 nm whereas there was less reduction at 254 nm for all the NOM preparations. The plot did not give clear information on the extent of reduction in $A_{254}$ as the culture fluids were diluted in 1/10 and so removal in terms of total carbon was calculated (Figure 4.3).

The initial and final colour (as $A_{446}$) of NOM 3 was much higher than for the other two NOM preparations even though the initial NOM concentrations in terms of total carbon were the same. The greatest decolourisation for all NOM preparations was obtained on day 3: 37% (NOM 3), 31% (NOM 1) and 29% (NOM 2). Losses in $A_{254}$ of 9% for NOM 3 and 3% and 1% for NOM 2 and NOM 1, respectively, occurred after five days (Figure 4.2).
Figure 4.2  $A_{446}$ and $A_{254}$ of NOM 1, NOM 2 and NOM 3, 100 mg C/L initial NOM concentration, *P. chrysosporium* ATCC 34541. ($A_{254}$ represents readings of 1/10 dilution of culture medium)

To elucidate NOM removed in terms of total carbon, NOM removals (converted to mg), measured at 446 nm and 254 nm, were calculated (Figure 4.3). The extent of NOM removal followed the trends: NOM 3 > NOM 1 > NOM 2 for $A_{446}$, and NOM 3 > NOM 2 > NOM 1 for $A_{254}$. The low reduction in $A_{254}$ indicates that the removal of conjugated double bonds (unsaturated aldehydes, phenols, aliphatic and aromatics) by these systems was negligible; consequently, little if any chemical change to the UV-absorbing components due to fungal activity or removal by adsorption occurred. The addition of NOM 1 and NOM 2 led to similar NOM removal, glucose consumption and biomass generation. In contrast, the fungus caused the removal of more NOM, higher glucose consumption and produced more biomass for NOM 3.

It was observed that the fungal pellets appeared brownish in colour. This indicated that NOM molecules were bonded to the fungal mycelium and so adsorption of NOM to the biomass seemed to play a role in colour removal as found by Rojek (2003). As the removal of colour was greatest for NOM 3, for which the biomass was greatest, and the fungal pellets were uniform in colour for all three NOM preparations, the major mechanism for NOM removal appeared to be via adsorption. This concurs with the previous proposal that NOM removal by biosorption occurred due to the pH drop (Rojek 2003) as low pH supports the binding of humic acid components to the fungal cell wall surface (Zhou & Banks 1991). In addition, the degree of removal of NOM was directly related to the amount of biomass produced due to the greater number of adsorption sites.
To better understand the components of the three NOM preparations, they were fractionated according to the method of Chow et al. (2004). This was to provide information on which types of compounds are removed in the treatment, as was mentioned by Yanagi et al. (2003) where the extent of decolourisation of humic acids varied with different chemical properties.

### 4.2 Fractionation of the MIEX™ NOM Preparations

The three NOM (100 mg C/L) preparations were separated into four fractions: VHA, SHA, CHA and NEU to establish the types of organic compounds present (see Section 3.6.9). The relative proportions of DOC, $A_{446}$ and $A_{254}$ in each fraction were determined. The proportion of each fraction in Figure 4.4 is an average of duplicate determinations, and the values of the duplicates varied only by ±1%.

The three preparations were dominated by hydrophobic acids such that VHA > SHA > CHA > NEU (Figure 4.4). NOM 1 and NOM 2 exhibited almost identical proportions of these fractions, but those in NOM 3 were markedly different. NOM 3 had the highest hydrophobic content with 69% VHA and 17% SHA. The hydrophilic neutral fraction was relatively small for all the preparations. This is because the MIEX™ NOM concentrates were obtained from the resin regeneration process, and constitute only approximately 80% of the NOM originally present in the water since the MIEX resin is an anionic exchanger and so cannot remove the neutral components of NOM (Slunjski et al. 2000).
Figure 4.4  The proportions of each fraction in the three NOM preparations: (A) NOM 1, (B) NOM 2 and (C) NOM 3 solutions. (N = 2; i.e., number of times each was determined)

It has been shown that NOM fractions contain a wide range of compounds and the types of compounds in each fraction are dependent on the water source. The typical classes of compounds in each fraction are summarised in Table 2.3 (Swietlik et al. 2004). The hydrophobic fractions are inclined to possess greater aromaticity than the hydrophilic fractions; therefore NOM 3 had the highest proportion of conjugated aromatic and high molecular weight compounds as supported by the HPSEC chromatograms in Figure 4.7(A).

The \( A_{446} \) (Figure 4.5) and \( A_{254} \) (Figure 4.6) for each fraction were measured to determine the colour and the UV-absorbing components, respectively, in the different NOM preparations, to give information on specific organic groups, rather than just the total dissolved organic species. The molecular weight distributions were then compared using HPSEC (Figure 4.7).

4.2.1  Comparison of \( A_{446} \) of the NOM fractions

Colour, as \( A_{446} \), was mainly contributed by the hydrophobic fractions, particularly the VHA fraction for which NOM 3 had the highest value (Figure 4.5A). The hydrophilic fractions (CHA and NEU) contributed little colour for all three NOM preparations. Thus the greatest removal of colour in the fermentation for NOM 3 (Figure 4.3) was due to its higher VHA content.

To better demonstrate the relative colour of each fraction in the NOM preparations, the \( A_{446} \) values were normalised against the DOC concentration of each fraction (\( A_{446}/\text{DOC} \)) (Figure 4.5B). It is clear that the specific \( A_{446} \) values for the VHA and SHA fractions of NOM 3 were higher than for NOM 1 and NOM 2. The decolourisation of NOM must be due to the breaking of bonds and/or adsorption of the VHA and SHA fractions of NOM as the specific values for
the CHA and NEU fractions were relatively low. This also explained the higher colour removal by *P. chrysosporium* from NOM 3 as being due to the greater hydrophobic content.

**Figure 4.5** \(A_{446}\) and \(A_{446}/DOC\) of the fractions of the three NOM preparations.

### 4.2.2 Comparison of \(A_{254}\) of the NOM fractions

The VHA fractions were the most intensely UV absorbing, with NOM 3 having the highest and NOM 1 and NOM 2 preparations displaying similar UV-absorbing contents (Figure 4.6A). The UV-absorbing species in the hydrophilic fractions were mostly in the CHA fraction; the NEU fractions showed very low \(A_{254}\) values.

**Figure 4.6** \(A_{254}\) and \(A_{254}/DOC\) of the fractions of the three NOM preparations.

However when the normalised \(A_{254}\) values were calculated, the VHA fraction in NOM 3 had relatively lower UV-absorbing content per unit DOC than NOM 1 and NOM 2. In contrast, the UV-absorbing content of the SHA fraction in NOM 3 was relatively higher. The \(A_{254}\) and also the normalised \(A_{254}\) of the NEU fractions were low for all three NOM preparations (Figure 4.6B). The higher total normalised \(A_{254}\) of the hydrophobic fractions (VHA + SHA)
for NOM 3 is consistent with the higher SUVA for this preparation and thus indicative of greater aromaticity. In contrast, the three NOM preparations had low total normalised $A_{254}$ of the hydrophilic fractions (CHA + NEU) and so did not contain high aromatic nor conjugated compounds in the hydrophilic fractions. Therefore, NOM 3 was considered to be composed largely of aquatic humic substances and a relatively higher content of hydrophobic and aromatic compounds compared with NOM 1 and 2, which is consistent with the high SUVA for this preparation (Table 3.1).

As the $A_{254}$ for all three NOM preparations was largely associated with the VHA and SHA fractions, reduction in $A_{254}$ in the presence of *P. chrysosporium* is considered to correspond to breaking of the UV-absorbing bonds in or removal of these fractions. To elucidate the breakdown products, GC-MS can be applied to fully establish bond breakage. Again, the greater removal of NOM 3 (Figure 4.3) was attributed to the higher VHA and SHA contents, although some CHA fraction may also be involved.

### 4.2.3 Comparison of molecular weight distribution of the NOM fractions

The molecular size distributions of the UV-absorbing components of the ‘whole’ NOM and each NOM fraction for the three NOM preparations were investigated (Figure 4.7). NOM 1 and NOM 2 had very comparable molecular weight distribution with a large peak at ~1500 Dalton and another at 400 Dalton (Figure 4.7A). NOM 3 had peaks at almost similar apparent molecular weights, with an additional peak at 1000 Dalton, and it exhibited some higher molecular weight compounds.

The UV-absorbing species in all the NOM preparations were mostly in the hydrophobic fractions (VHA and SHA). The hydrophobic fractions contained molecules of apparent molecular weight $>$2000 Dalton for the three NOM preparations, but this was not so for the CHA fractions. The molecular weight distribution of each fraction was very similar for NOM 1 and NOM 2; NOM 3 had a very different pattern for all fractions. It should be noted that the NEU fractions were very small for all and so no trends were apparent.

The VHA fraction gave two peaks for all the NOM preparations: at apparent molecular weights of ~1500 Dalton and 400 Dalton (Figure 4.7B). The absorbance at the peak of ~1500 Dalton for NOM 3 was lower than for NOM 1 and NOM 2, however, it contained more compounds with apparent molecular weight $>$2000 Dalton.
The SHA fraction comprised markedly fewer high molecular weight UV-absorbing compounds than the VHA fraction for the three NOM preparations. NOM 3 possessed two peaks in the molecular weight range of 1000-2000 Dalton but with lower absorbance compared with the VHA fraction. There was only one peak in the SHA fraction for NOM 1 and NOM 2, which was at ~1500 Dalton (Figure 4.7C).

The CHA fraction for NOM 3 was relatively lower than for NOM 1 and NOM 2. All NOM preparations had a peak at ~1500 Dalton and an additional peak at 1000 Dalton for NOM 3 (Figure 4.7D). Their UV-absorbing contents were very small compared to the hydrophobic fractions, especially in NOM 3.

There was little, if any, UV-absorbing species in the NEU fraction for all the NOM preparations (Figure 4.7E) as the most UV-absorbing compounds in the hydrophilic fractions (CHA and NEU) were in the CHA fraction.

The three NOM preparations contained less non-humic (hydrophilic) than humic (hydrophobic) substances due to the lower contents of the UV-absorbing species in the hydrophilic fractions, especially in NOM 3, than the hydrophobic fractions. Possible constituents of the hydrophilic fractions would be hydrophilic base compounds such as amphoteric proteinaceous materials containing aliphatic amino acids, amino sugars, peptides and proteins (Table 2.3) as the NOM preparations were highly alkaline (pH 7.2-8.3) (Table 3.1). Polysaccharides, hydrophilic neutral compounds, were unlikely to be present in the NOM preparations as the NEU fraction was very small (Figure 4.4).

All the NOM preparations most likely contained fulvic acids and humic acids with NOM 3 having the highest humic acid content as it was highly alkaline and dark brown in colour, and had more high molecular weight compounds (>2000 Dalton). This agrees with the hypothetical relationships for chemical characteristics of humic substances (Figure 2.3) where humic acids showed higher colour intensity and molecular weight, and lower acidity than fulvic acids. In addition, Howe and Clark (2002) reported that the molecular weight distribution for aquatic humic acid was 2000-5000 Dalton, where NOM 3 showed the highest content (Figure 4.7A). NOM 3 contained both aliphatic and aromatic high molecular weight compounds with less for NOM 1 and NOM 2 as NOM 3 had the highest total normalised A254 of the hydrophobic fractions (Figure 4.6B), which is consistent with the highest SUVA value for this preparation (Table 3.1) and thus indicative of greater aromaticity.
Figure 4.7 HPSEC chromatograms for the (A) ‘whole’ NOM, (B) VHA, (C) SHA, (D) CHA and (E) NEU fractions for all NOM preparations.
The weight average molecular weight (M$_w$) and the number average molecular weight (M$_n$) were calculated so as to compare the variation in molecular weight and the extent of polymerisation (Figure 4.8). M$_w$ for NOM 1 and NOM 2 were similar, that of NOM 3 was higher. M$_n$ for all NOM preparations was comparable. Polydispersity, calculated as the ratio of M$_w$: M$_n$, is a measure of the extent of polymerisation. The polydispersity of NOM 3 was approximately 20% higher than that NOM 1 and NOM 2. These results were consistent with the previous observations where NOM 3 had the highest content of high molecular weight compounds and greatest colour intensity. These findings were also in agreement with the postulated relationships reported by Weber (2001) (Figure 2.3), where the degree of polymerisation increases with the intensity of colour and molecular weight.
4.3 Molecular Size Distribution of the NOM after Treatment with *P. chrysosporium* ATCC 34541

The molecular weight distribution of the UV-absorbing species for the three NOM preparations after five days treatment with *P. chrysosporium* (Section 4.1) was determined using HPSEC (Figure 4.9).

A small shift from higher to lower molecular weight for the NOM remaining after treatment with *P. chrysosporium* was observed for all NOM preparations (Figure 4.9). NOM 3 showed a slightly greater shift to lower molecular weight following the treatment. The removal of the high molecular weight compounds was accompanied by the accumulation of the low molecular weight species, presumably due to some breakdown by the fungus, especially for the NOM 3 preparation. Blondeau (1989) also obtained reduction in the high molecular weight range but without accumulation of low molecular weight species after the fungal treatment. However, adsorption also contributed to the removal of the coloured high molecular weight fractions as the fungal pellets turned brown as found by Rojek (2003).

All the NOM preparations exhibited a decrease in the UV absorbance for the molecular weight fraction >2000 Dalton. This was largely due to the decrease in the high molecular weight hydrophobic fractions and was most marked for NOM 3 as only the hydrophobic fractions exhibited apparent molecular weight >2000 Dalton (Figure 4.7A & B). For the cultures with NOM 1 and NOM 2 there was almost no shift in the apparent molecular weight range 1000-2000 Dalton, and there was a similar reduction in absorbance for the peak at ~1500 Dalton. In contrast, there was a slight shift in apparent molecular weight in the range 1000-2000 Dalton and some decreases in absorbance for the culture with NOM 3.

*P. chrysosporium* seemed to preferentially remove the VHA fraction, and so was most effective for the NOM preparation with the highest VHA content. However, this could not be demonstrated by fractionation of the NOM-containing medium after growth of the fungus as it contained glucose, metabolic products and salts, which would have interfered with the function of the resins.
Figure 4.9  HPSEC chromatograms for the three NOM preparations incubated with *P. chrysosporium* in Waksman medium, control-NOM (medium plus NOM), control-*P. chry* 34541 (*P. chrysosporium* ATCC 34541 grown without NOM).
The weight average molecular weight (M<sub>w</sub>) and number average molecular weight (M<sub>n</sub>) for UV-absorbing components based on HPSEC were calculated after five days treatment of the three NOM preparations with <i>P. chrysosporium</i>. The greatest reduction in M<sub>w</sub> and M<sub>n</sub> was obtained for the culture containing NOM 3. The original values of M<sub>w</sub> of 10,188 (NOM 3), 8626 (NOM 2) and 8514 (NOM 1) were reduced by 3%, 2% and 2%, respectively. There was a similar trend of reduction in M<sub>n</sub> for all the NOM preparations (Figure 4.10).

The NOM 3 preparation was selected for further investigation as this batch showed greatest NOM removal, had the greatest colour, aromaticity and the highest SUVA, and exhibited the greatest change in the high molecular weight compounds due to its high proportion of the hydrophobic fractions and so provided a suitable material to further investigate NOM removal.

![Figure 4.10 Reduction in weight average molecular weight (M<sub>w</sub>) and number average molecular weight (M<sub>n</sub>) after the treatment of the NOM preparations with <i>P. chrysosporium</i> ATCC 34541.](image)

**Figure 4.10** Reduction in weight average molecular weight (M<sub>w</sub>) and number average molecular weight (M<sub>n</sub>) after the treatment of the NOM preparations with <i>P. chrysosporium</i> ATCC 34541.

### 4.4 Selection of Medium

The aim of this study was to develop a system for the bioremediation of concentrated NOM in a simple medium. As removal of NOM from Waksman medium by <i>P. chrysosporium</i> ATCC 34541 was low, two other media were tested to see if NOM removal could be increased with different C:N ratios.

The effects of three different growth media: Waksman, Fahy and Fujita medium, on NOM decolourisation by <i>P. chrysosporium</i> were established. Carbon starvation (1.0 g/L glucose)
did not trigger a degradation process (Rojek et al. 2004) and so Waksman medium with 2.0 g/L glucose was selected. These media were chosen as they showed a wide range of initial glucose and nitrogen concentrations, thus giving different ratios. C:N ratio has been considered as a better predictor of lignin degradation than the absolute levels of carbon and nitrogen (Reid 1979). The C:N ratios of each medium are stated in Table 4.1.

Table 4.1 The C:N ratios of the different media used.

<table>
<thead>
<tr>
<th>Medium</th>
<th>C:N ratios</th>
<th>Composition (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>Waksman</td>
<td>6</td>
<td>2.0</td>
</tr>
<tr>
<td>Fahy</td>
<td>76</td>
<td>25.0</td>
</tr>
<tr>
<td>Fujita</td>
<td>114</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Growth medium (200 mL) with the addition of NOM 3 preparation (final concentration of 100 mg C/L) was inoculated with a spore suspension of *P. chrysosporium*. The fungal cultures were then incubated at 36°C and 130 rpm. All cultures were performed in duplicate.

![Graph A](image1.png) ![Graph B](image2.png)

**Figure 4.11** Decolourisation and glucose consumption in the different growth media containing 100 mg C/L NOM and *P. chrysosporium* ATCC 34541.

Maximum colour removal over the 14-day incubation period occurred on day 10 with 41%, 32% and 29% removal for Waksman, Fahy and Fujita medium, respectively (Figure 4.11A). Although the extent of decolourisation for Fahy and Fujita medium was lower than for Waksman medium, they consumed more glucose (2.54 g/L and 3.54 g/L versus 1.14 g/L for Waksman medium by day 10) (Figure 4.11B). Decolourisation started to decline after day 10,
yet the fungus continued to consume glucose, especially in Fahy and Fujita medium. The decrease in decolourisation may be due to desorption of the NOM from the fungal cell wall.

Incubation of *P. chrysosporium* in Waksman medium, which contained the lowest glucose content, generated the highest biomass and achieved the greatest NOM removal. Fahy medium had slightly higher NOM removal and biomass production compared with Fujita medium (Figure 4.12). The fungal pellets generated in the three culture media had a brown appearance indicating involvement of NOM adsorption in the decolourisation of NOM. In a study by Carliele *et al.* (2001), it was demonstrated that carbon and nitrogen ratios of 10:1 or less were optimum for the growth of fungi. This is consistent with the present results where the lower the C:N ratio, the greater biomass formation and thus higher NOM removal achieved. Rojek (2003) also demonstrated that the removal of NOM increased with decreased C:N ratio although a different NOM preparation was used. However, as a different NOM preparation was employed there was a little lower decolourisation (41% cf. 47%) even though a little lower C:N ratio was used (6 cf. 7.90) compared with the results of Rojek (2003).

![Figure 4.12](image)

**Figure 4.12**  Dry weight biomass (mg) and NOM removal in terms of $A_{446}$ (converted to mg/L) by *P. chrysosporium* ATCC 34541 in different culture media after 14 days.

The synthesis efficiency (yield), which is dried mycelium weight divided by weight of carbon source used, for Waksman, Fahy and Fujita medium was 0.36, 0.13 and 0.10, respectively. Thus, it is more efficient to use the medium with the lowest glucose content (Waksman) as it was apparently not limiting to growth, and gave the greatest NOM removal both in absolute terms and per unit of glucose consumed. Thus, Waksman medium with the C:N ratio of 6 was selected as the growth medium in the following experiments.
4.5 Selection of Organism

As removals of NOM with *P. chrysosporium* ATCC 34541 were low, and little biodegradation occurred, this organism was compared with *P. chrysosporium* strain ATCC 24725, another species of white-rot fungus (*Trametes versicolor* ATCC 7731), and three strains of yeast (*Saccharomyces* species 1, 2 and 3), which had been isolated from a bottle of MIEX concentrate. As the aim of this study was to develop a simple system for NOM removals from solution within a short time frame, 5 or 7-day fermentations were investigated.

Waksman medium (200 mL) with the addition of the NOM 3 preparation (final concentration of 100 mg C/L) was inoculated with either 10 mL spore suspension of white-rot fungi or a loopful of yeast from a MEA culture. The cultures were then incubated at 130 rpm and 36°C (white-rot fungi) or 30°C (the yeast). The yeast used had been shown to decolourise NOM solutions in a preliminary experiment conducted in the laboratory at RMIT.

Cultures with white-rot fungi and the yeast were incubated for five days and seven days respectively and monitored for decolourisation of NOM (Figure 4.13). Of the white-rot fungi, *T. versicolor* achieved the highest colour removal. Decolourisation increased rapidly until it reached a plateau on day 3, at which 59% colour removal was attained. The two strains of *P. chrysosporium* displayed comparable colour reduction (37%), which plateaued after day 2 (Figure 4.13A). *Saccharomyces* sp. 1 and 3 seemed to have little potential for the removal of NOM (Figure 4.13B). *Saccharomyces* sp. 2 gave similar high colour reduction to *T. versicolor*, however, the specific removal values differed markedly: 0.055 compared to 0.089 mg NOM/mg biomass, respectively.

![Figure 4.13 Decolourisation of the NOM (100 mg C/L initial concentration) in Waksman medium by (A) white-rot fungi and (B) *Saccharomyces* sp.](image_url)
Figure 4.14 Biomass dry weight (mg) and glucose consumptions (g/L) of Saccharomyces spp. 1-3 and T. versicolor, Waksman medium with 2 g/L initial glucose content.

Saccharomyces sp. 2 and T. versicolor consumed comparable amounts of glucose with 0.031 mg NOM removed per unit glucose consumed. Saccharomyces sp. 1 and 3 gave 0.015 and 0.013 mg NOM removal per unit glucose consumed, respectively. Saccharomyces sp. 2 produced the highest biomass whereas Saccharomyces sp. 1 provided the least biomass (Figure 4.14).

The colour removal by the Saccharomyces species was attributed predominantly to adsorption as indicated by the deep brown colouration of the biomass for all species (see Figure 4.15 for example), whereas the T. versicolor was light brown in colour (Figure 4.18B). The yeast removed little UV-absorbing NOM as shown in the HPSEC chromatograms in Figure 4.16; there was only a small reduction in the high molecular weight range and no formation of lower molecular weight materials. This was consistent with the NOM removal measured as reduction in $A_{446}$ (Figure 4.13B) being primarily due to adsorption rather than biodegradation. This biosorption, combined with the difficulty experienced in getting consistent results with the yeast, led to no further investigation of the yeast.
Figure 4.15  Biomass of *Saccharomyces* sp. 2 (A) incubated in the absence of NOM and (B) after incubation with 100 mg C/L NOM.

Figure 4.16  HPSEC chromatograms for NOM remaining after treatment with *Saccharomyces* sp. 2 in Waksman medium for seven days (control-NOM: culture in the absence of the yeast; control-*Saccharomyces* sp. 2: culture in the absence of NOM).

Plots of NOM removal ($A_{446}$ and $A_{254}$, converted to mg), glucose consumption (g/L) and biomass generated (mg) for the three white-rot fungi were constructed for comparison (Figure 4.17). *T. versicolor* attained the highest reduction in colour and UV-absorbing components whereas the two strains of *P. chrysosporium* exhibited similar NOM removals. NOM removals in terms of colour and UV-absorbing species achieved by *T. versicolor* were approximately 62% and 80% greater, respectively, than for both strains of *P. chrysosporium*. The ratios of $A_{254}/A_{446}$ removal for the two strains of *P. chrysosporium* were similar (0.25) whereas for *T. versicolor* it was slightly higher (0.29).
*T. versicolor* consumed all glucose supplied, unlike both strains of *P. chrysosporium* (Figure 4.17). The yields \((Y_{xs})\) for *P. chrysosporium* ATCC 34541 and ATCC 24725 and *T. versicolor* were 0.32, 0.29 and 0.35, respectively, indicating that *T. versicolor* was more efficient in generating biomass under these conditions. It was observed that the *P. chrysosporium* pellets were deep brown (Figure 4.18A) whereas the *T. versicolor* pellets were slightly lighter in colour (Figure 4.18B).

**Figure 4.17** Comparison of NOM removal (as mg, converted from \(A_{446}\) and \(A_{254}\)), glucose consumption and biomass for the three white-rot fungi at initial concentrations of 2 g/L glucose and 100 mg C/L NOM after five days.
Figure 4.18  Biomass of (A) *P. chrysosporium* ATCC 34541 and (B) *T. versicolor* in the absence of NOM (top), and after five days incubation with 100 mg C/L NOM (bottom).

Figure 4.19  HPSEC chromatograms for NOM treated with *P. chrysosporium* ATCC 34541 and *T. versicolor* ATCC 7731 (control-NOM: culture in the absence of the fungi; control-*P. chry* 34541 or control-*T. ver* 7731: culture in the absence of NOM).
HPSEC analysis was performed to determine any changes in the molecular weight distribution of the UV-absorbing species of the NOM remaining after treatment. A shift from high molecular weight towards lower molecular weight species was observed for both fungal species and was most marked for *T. versicolor*. *T. versicolor* gave greater degradation of the high molecular weight compounds. The absorbance of the peak at ~1500 Dalton for both *P. chrysosporium* and *T. versicolor* cultures was reduced by approximately 0.03 cm\(^{-1}\) and 0.10 cm\(^{-1}\), respectively, after NOM treatment. The absorbance of the low molecular weight compounds for both cultures was increased after the treatment, indicating that biodegradation of NOM had occurred to form a pool of low molecular weight compounds; this was greater for the *T. versicolor* culture. There were two new peaks formed at 700 and 1000 Dalton after the treatment with *T. versicolor*, again indicating the greater breakdown of the high molecular weight NOM, and suggesting a possible mechanism of sequential breakdown of the larger NOM molecules via molecules of intermediate size (Figure 4.19).

![Figure 4.20 Weight average molecular weight (M_w) and number average molecular weight (M_n) for the NOM (control) and the NOM remaining after five days treatment with *P. chrysosporium* ATCC 34541 or *T. versicolor* ATCC 7731.](image)

M\(_w\) and M\(_n\) were calculated after five days treatment of the NOM with *P. chrysosporium* and *T. versicolor*. The greatest reductions in M\(_w\) and M\(_n\) were obtained for the *T. versicolor* culture, 25% and 30%, respectively. There were lower reductions in M\(_w\) and M\(_n\) for the *P. chrysosporium* culture, approximately 13% (Figure 4.20). This is consistent with the outcomes of the molecular weight distribution (Figure 4.19) for the NOM treated with *T. versicolor* where there was apparent sequential breakdown of the larger NOM molecules via molecules of intermediate size.
It was considered that the extracellular phenoloxidase enzymes (LiP, MnP and Lac) may have been involved in the biodegradation of NOM by *T. versicolor*. Consequently, a spot test on a colony on an agar plate was undertaken.

A reddish brown zone was visible on the *T. versicolor* colony when 0.02% guaiacol was added indicating the presence of laccase as laccase catalyses the oxidative polymerisation of guaiacol to form brown tetraguaiacone (Figure 4.21).

![0.02% guaiacol](image1.png)

**Figure 4.21** Reaction of guaiacol on *T. versicolor* agar plate colony indicating presence of the laccase enzyme.

To investigate this further the activity of LiP, MnP and Lac for the three white-rot fungi was assayed on day 3 (fermentations in Figure 4.13A) to determine if they were involved in the removal of the NOM (Figure 4.22).

![Enzyme activity graph](image2.png)

**Figure 4.22** Activity of the extracellular phenoloxidase enzymes in 3-day cultures of the three white-rot fungi.
LiP was the main enzyme secreted by *P. chrysosporium*, where strains ATCC 34541 and ATCC 24725 produced 2.4 U/L and 1.5 U/L LiP, respectively; very-low activities of MnP and Lac were found. Interestingly, Lac activity was detected in the cultures of *P. chrysosporium* under the conditions used. Moreover, it is generally accepted that this fungus does not produce laccase. The detected activity may be attributed to the non-specificity of the substrate (guaiacol) used for the enzyme assays. The very low level of LiP activity detected for the *P. chrysosporium* cultures was most likely due to low levels of dissolved oxygen as the system requires high dissolved O$_2$ for lignin decomposition (Kirk *et al.* 1978). There was little MnP activity, this may have been due to low levels of Mn$^{2+}$ in the liquid cultures inhibiting MnP production, as Mn$^{2+}$ can stimulate MnP production and functions as a substrate for MnP (ten Have & Teunissen 2001). However, Rojek *et al.* (2004) reported that addition of Mn$^{2+}$ (1.2 mg/L final concentration) did not result in greater decolourisation and so MnP may not be involved in the process or sufficient Mn was already present in the culture medium.

For *P. chrysosporium*, LiP was the major enzyme that caused biodegradation of the NOM. This enzyme has the ability to partly depolymerise and cleave C$_\alpha$-C$_\beta$ linkages in side chains. A study by Dehorter and Blondeau (1992) reported that *P. chrysosporium* produced 0.4 U/mL and 1.1 U/mL LiP and MnP, respectively, to achieve high decolourisation (~60%) of 0.05% soil humic acids after a 5-day treatment. Another study stated that 1.2 U/mL LiP was produced by *P. chrysosporium* for pentachlorophenol (PCP) degradation with no mention of MnP and Lac activities (Shim & Kawamoto 2002). However, the small magnitude of LiP activity (0.002 U/mL) for the two strains of *P. chrysosporium* in the present study, compared with the findings of other researchers, probably led to the low degree of NOM removal by biodegradation. Thus, this also verified that *P. chrysosporium* removed the NOM primarily by adsorption as indicated by the deep brown colouration of the biomass (Figure 4.18A), rather than enzymatically oxidised via ligninolytic enzymes.

*T. versicolor* had high Lac activity compared with LiP and MnP activity. The high NOM removal attained by *T. versicolor* was attributed to the laccase, although the pellets were light brown in colour. The HPSEC pattern for *T. versicolor* suggests that the high Lac activity allowed it to break down a higher proportion of the high molecular weight compounds than *P. chrysosporium* and so form lower molecular weight molecules including two peaks at 700 and 1000 Dalton. Furthermore, this also indicates that laccase was able to break different types of bonds compared with LiP, such as demethylation of methoxy groups and catalysing C$_\alpha$-C$_\beta$ cleavage of phenolic moieties via phenoxy radicals (Kawai *et al.* 1988).
From the results, *T. versicolor* has more potential for breaking down the NOM while *P. chrysosporium* removed the NOM primarily by adsorption under the conditions studied. Consequently, *T. versicolor* was selected for further investigation. Enhancement of NOM removal was studied by determining the effects of supplements, pH, temperature and initial NOM concentration on fungal growth and extracellular enzyme activities, as described in Chapter 5.
Chapter 5  Biodegradation of NOM by *Trametes versicolor*

Of the organisms investigated in Chapter 4, *T. versicolor* was the most effective for the biodegradation of NOM and gave the greatest NOM removal under the conditions used. Therefore, further investigation was undertaken into the conditions to improve growth and enhance the extracellular enzyme activities of *T. versicolor* to enhance NOM removal. This was followed by investigation of the enzymatic treatment of the concentrated NOM *in vitro*.

5.1  Improving NOM Removal by Altering Culture Conditions

To improve NOM removal from solution by *T. versicolor* the impacts of incubation temperature, carbon source level, type of inoculum and NOM concentration were investigated.

5.1.1  Incubation temperature

As most studies used 30°C (Mehna *et al.* 1995, Swamy & Ramsay 1999, Lorenzo *et al.* 2002, Rancañó *et al.* 2003, Dodor *et al.* 2004) as the incubation temperature for *T. versicolor* cultures, a comparison of the effect of the temperatures 30°C and 36°C on NOM removal was undertaken.

Cultures containing 200 mL Waksman medium (2 g/L glucose) and filter sterilised NOM (final concentration of 100 mg C/L) were prepared and inoculated with fungal spore suspensions, and incubated at 30°C or 36°C and 130 rpm for nine days. All fermentations were performed in duplicate. Data points correspond to the average of replicates.

The pH and glucose consumption trends for the two conditions differed markedly (Figure 5.1). The pH dropped to almost 3 on day 2 for the cultures at 36°C whereas this occurred on day 4-5 for the cultures at 30°C. The cultures incubated at 36°C consumed all the glucose within the first three days. At 30°C, glucose consumption was slow until day 5, after which there was a major increase such that it was exhausted by day 9.

The performance of the fungus in terms of NOM removal measured at both 446 nm and 254 nm was greater at the higher than the lower temperature over the first three days, after which it plateaued. This plateau coincided with the exhaustion of the glucose, where 50% and 20% reductions in $A_{446}$ and $A_{254}$, respectively, were obtained for the cultures at 36°C. Rapid NOM
removal and then cessation coinciding with the rapid and then cessation of glucose consumption suggests that either NOM removal is linked with glucose consumption/growth and/or to biosorption at 36°C. In contrast, there were only slight reductions in both $A_{446}$ and $A_{254}$ until day 4 at 30°C, which then increased to give 73% and 55% reductions in both $A_{446}$ and $A_{254}$, respectively, by day 9. The slow reductions within the first four days at 30°C were probably due to initial adsorption or uptake of NOM, which was followed by enzyme induction and thus breakdown of NOM.

$30^\circ C$

30°C

$36^\circ C$

36°C

Figure 5.1 History plots for *T. versicolor* cultures containing 100 mg C/L NOM incubated at 30°C and 36°C, Waksman medium 2 g/L initial glucose.

The NOM removal efficiency in terms of colour was higher at 36°C than at 30°C, 0.075 cf. 0.062 mg NOM removed/mg biomass (Figure 5.2). The high specific colour removed at 36°C was at least partially due to adsorption as indicated by the brown colouration of the biomass (Figure 5.3B) compared to the cultures at 30°C, where the pellets were cream in colour (Figure 5.3A). This supports the findings in Figure 5.1 where biosorption was partially involved in the NOM removal at 36°C. The specific removal of UV-absorbing components at 30°C (0.046 mg NOM removed/mg biomass) was markedly better than that at 36°C (0.030 mg NOM removed/mg biomass). The ratio $A_{254}/A_{446}$ at 30°C was higher than at 36°C, 0.75 cf. 0.40, suggesting that the fungus removed/cleaved a higher proportion of molecules containing conjugated bonds at the lower temperature.

Less biomass was produced at 36°C than at 30°C (Figure 5.2), with the yields ($Y_{x/s}$, biomass per unit glucose consumed) of 0.33 and 0.60 respectively. This further indicates that 30°C is a
more appropriate incubation temperature for *T. versicolor*. Growth of organisms at elevated temperatures results in the inhibition of fungal growth and possibly inhibition of or denaturation of enzymes.

![Graph showing NOM removals (as mg, converted from $A_{446}$ and $A_{254}$) and biomass produced (mg) in *T. versicolor* cultures at 30°C and 36°C.](image)

**Figure 5.2** NOM removals (as mg, converted from $A_{446}$ and $A_{254}$) and biomass produced (mg) in *T. versicolor* cultures at 30°C and 36°C.

![Images of *T. versicolor* biomass incubated at (A) 30°C and (B) 36°C in the absence of (top) and presence of (bottom) NOM after nine days incubation, 100 mg C/L NOM.](image)

**Figure 5.3** Biomass of *T. versicolor* incubated at (A) 30°C and (B) 36°C in the absence of (top) and presence of (bottom) NOM after nine days incubation, 100 mg C/L NOM.

There are clear differences in the behaviour and performance of the organism at the different temperatures. The behaviour of *T. versicolor* at 36°C was very similar to that of *P.*
*chrysosporium* (Figure 4.1), in that the fast NOM removal rate over the first three days followed by a plateau indicated that the mechanism of NOM decolourisation was primarily biosorption, as confirmed by the brown colouration of the fungal pellets. This is consistent with the findings of Rojek (2003), who reported the same trends for *P. chrysosporium* ATCC 34541 and established that adsorption contributed 60-70% of the overall removal of NOM. On the contrary, the initial slow and then higher rate of NOM removal with *T. versicolor* at 30°C was attributed to the production of oxidative enzymes during secondary metabolism. The lack of brown colouration of the fungal pellets (Figure 5.3A) supports this premise of NOM removal by biodegradation rather than adsorption. The trends in reduction in $A_{446}$ and $A_{254}$ can be related to the activities of the extracellular phenoloxidase enzymes (Figure 5.4).

![Figure 5.4](image_url)  
**Figure 5.4** Activity of the extracellular phenoloxidase enzymes of the *T. versicolor* cultures incubated at 30°C and 36°C.

The low production of the phenoloxidase enzymes at 36°C was very apparent. The activity of the LiP, MnP and Lac enzymes was much higher at 30°C, as was the removal of NOM. This, in combination with the different patterns for pH, $A_{446}$ and $A_{254}$ reductions and glucose consumption indicates that these enzymes played a major role in NOM removal. The reduction in $A_{254}$ indicates that conjugated bonds and aromatic rings were enzymatically broken. However, the low activity of LiP in both cultures suggests that this enzyme plays only a minor role in NOM removal for *T. versicolor* under the conditions employed. The ratio of LiP to Lac activity for both cultures was similar (~0.075), while the ratio of MnP to Lac activity at 30°C was markedly higher than that at 36°C, 0.358 cf. 0.082, suggesting that MnP and Lac are the main enzymes involved in the removal of NOM at 30°C under the conditions studied. The high activities of MnP and Lac obtained in this culture would be responsible for
the cleavage of aromatic rings, conjugated bonds and Cα-Cβ bonds in phenolic moieties, as well as catalysing alkyl-aryl cleavage in the NOM structures. The lignin-degrading enzymes may also be involved in the breakdown of β-O-4 and β-1 linkages that occur within the lignin polymer.

Although two studies (Blondeau 1989, Dehorte & Blondeau 1992) reported that LiP and MnP were associated with humic acid degradation, the resistance of humic substances to microbial decolourisation is largely related to the differences in their chemical structures and is microbial species dependent, as suggested by Yanagi et al. (2002), and is dependent upon the culture conditions. Moreover, Dehorter and Blondeau (1992) established that MnP rather than LiP was the major enzyme involved in the microbial degradation of different concentrations of humic acid with T. versicolor. The high levels of MnP and Lac observed for T. versicolor at 30°C are consistent with the findings of Galliano et al. (1991), who reported that these enzymes worked synergistically in the degradation of lignin. They reported that when these two enzymes were isolated and purified from Rigidoporus lignosus, neither was able to solubilise lignin. However, degradation of lignin occurred when the two enzymes were added to the reaction medium simultaneously.

These findings support the suggestion that removal of NOM by T. versicolor incubated at 30°C was mainly due to enzymatic breakdown whereas at 36°C it was probably removed by different mechanisms such as: chemical and physical sorption, metabolically dependent sorption and accumulation, and biodegradation as reported by Rojek (2003) in relation to the NOM removal with P. chrysosporium.

The impact of the NOM-degrading enzymes on the molecular weight distribution of the UV-absorbing species for the NOM remaining after nine days treatment with T. versicolor at 30°C and 36°C was determined (Figure 5.5).

A shift from high molecular weight towards lower molecular weight species was observed for both temperatures. It was observed that low molecular weight compounds for the culture at 36°C were formed from the breakdown of the high molecular weight compounds. However T. versicolor at 30°C was markedly more effective in removing and converting the high molecular weight UV-absorbing species to lower molecular weight compounds. The fungus at 30°C was able to remove compounds of apparent molecular weight smaller than 1000 Dalton, and the first peak at 350 Dalton remained similar or reduced slightly, indicating that either the
low molecular weight species were produced (from the breakdown of the high molecular weight molecules) and were removed simultaneously probably by metabolism or, that the low molecular weight species were not UV-absorbing. As noted previously, the ability of *T. versicolor* to degrade NOM was greater at 30°C than at 36°C probably due to its greater production of NOM-degrading enzymes, especially MnP and Lac, where MnP is presumably responsible for the removal of low molecular weight species. Thus, MnP and Lac may act synergistically in the enzymatic breakdown of medium molecular weight (500-2000 Dalton) fulvic acids and high molecular weight (2000-5000 Dalton) humic acids.

Figure 5.5 HPSEC chromatograms for NOM remaining after treatment with *T. versicolor* incubated at 30°C and 36°C; controls represent fungal cultures grown in the absence of NOM.

The greater reduction in $M_w$ and $M_n$ for the *T. versicolor* cultures at 30°C further demonstrates that the biodegradation of NOM at lower temperature was markedly more effective than at higher temperature (Figure 5.6). A reduction of 40% in both $M_w$ and $M_n$ was achieved for *T. versicolor* at 30°C, suggesting that the NOM removal was via enzymic breakdown, which was probably followed by fungal metabolism.
5.1.2 Carbon source level

This experiment was performed as the glucose provided in the preceding experiment was exhausted after nine days at 30°C. A study by Rojek (2003) suggested that glucose exhaustion could have an inhibitory effect on NOM decolourisation by *P. chrysosporium* ATCC 34541. Thus a higher initial glucose concentration (5 g/L) was supplied to see if this would increase fungal growth, secretion of ligninolytic enzymes and NOM removal with *T. versicolor* at 30°C.

Waksman medium (200 mL) containing either 2 or 5 g/L glucose and 100 mg C/L NOM was inoculated with *T. versicolor* as spore suspensions and incubated at 30°C and 130 rpm for nine days. The history plot for the culture with 2 g/L glucose was obtained from Figure 5.1, the fermentation containing 5 g/L glucose was conducted in duplicate and the average values obtained.

The pH trends for both cultures at different initial glucose concentrations were comparable, where the pH dropped to 3 on day 4-5 (Figure 5.7). Although the pH dropped to 3, it did not seem to be associated with long-term adsorption of NOM to biomass when the phenoloxidase enzymes were active. The rate of glucose consumption was low initially and increased after day 5 for 2 g/L initial glucose, whereas it was greater before and then decreased after day 5 for 5 g/L initial glucose cultures.
As discussed in Section 5.1.1 microbial breakdown of NOM occurred in the *T. versicolor* culture with 2 g/L glucose at 30°C, as the rate of NOM removal (which was slow initially and then increased markedly after day 4) was correlated with the induction of NOM-degrading enzymes. With 5 g/L initial glucose content, there were only slight reductions in $A_{446}$ and $A_{254}$ for the first three days, greater reductions occurred during day 3-4, and then both absorbances stayed constant. The sharp absorbance drop occurred at the same time as the pH drop for the cultures with 5 g/L initial glucose, indicating possible biosorption of the NOM by the fungal cell walls. This was confirmed by the brown colouration of the biomass. The *T. versicolor* preferentially consumed glucose rather than NOM in the culture, as glucose is a more easily obtained source of carbon than NOM. If the fermentation were left longer, it is possible that once the glucose was depleted then the NOM would be utilised as a carbon source and so removed from solution and possibly from the surface of the biomass.

![Figure 5.7](image)

**Figure 5.7** History plots for *T. versicolor* cultures containing 100 mg C/L NOM incubated at 30°C, Waksman medium containing 2 g/L and 5 g/L initial glucose.

Increasing the glucose content to 5 g/L resulted in higher biomass production (Figure 5.8). The yields $(Y_{x/s})$ for the 2 g/L and 5 g/L glucose cultures were 0.60 and 0.65 respectively. Although the yield was higher for the culture with the higher initial glucose concentration, the fungus did not attack the NOM until the glucose was depleted. Higher glucose concentration led to lower NOM removal efficiencies in terms of $A_{446}$ and $A_{254}$, which were 0.026 and 0.012 mg NOM removed/mg biomass respectively. The ratio of $A_{254}$ to $A_{446}$ for the culture with higher glucose concentration was lower, 0.45 cf. 0.75, demonstrating that this culture removed a higher proportion of coloured than the conjugated/aromatic species.
Enzyme activities were lower in the presence of the higher glucose concentration (Figure 5.9). Lac activity was markedly lower, especially in relation to MnP activity, for the higher glucose concentration. The low levels of these enzymes in the culture resulted in the inability of the fungus to break down the NOM. These further support the argument that the enzymatic activity is enhanced upon nutrient starvation (as established in the culture with the lower initial glucose content), which is known to contribute to the secondary metabolism during which the white-rot fungi produce the ligninolytic enzymes, and thus resulting to higher enzymatic removal of NOM.

**Figure 5.8** NOM removals (as mg, converted from A$_{446}$ and A$_{254}$) and biomass produced (mg) in *T. versicolor* cultures containing 2 g/L and 5 g/L initial glucose.

**Figure 5.9** Activity of the extracellular phenoloxidase enzymes of the *T. versicolor* cultures containing 2 g/L and 5 g/L initial glucose.
5.1.3 Types of inoculum

Spore collection for *T. versicolor* was very tedious, as approximately 20-30 agar plates with an actively growing fungal colony were needed to achieve a concentration of $1.0 - 1.5 \times 10^5$ spores/mL in each culture. Hence a comparison of NOM removal using inoculation by spores and by plugs was undertaken as the latter is a much easier method.

Cultures containing 200 mL Waksman medium (2 g/L glucose) and sterilised NOM (final concentration of 100 mg C/L) were prepared and inoculated with the fungus as either spore suspensions or as three MEA plugs (each 1 cm$^2$) of *T. versicolor* and incubated at 30°C and 130 rpm for eight days. All fermentations were performed in duplicate.

The pH, glucose consumption, and $A_{446}$ and $A_{254}$ trends for both culture types were similar (Figure 5.10). With plugs the pH dropped to pH 3.5 cf. pH 3.0 for the spore culture. The fungus in the plug culture did not consume glucose for the first two days, as there was some readily assimilable carbon available in the MEA plugs. The major decrease in $A_{446}$ and $A_{254}$ for both cultures occurred after day 4, presumably due to the production and action of ligninolytic enzymes involved in the degradation of NOM. After eight days the biomass was collected and dried and it appeared that the biomass on the plugs and the biomass pellets formed were creamy brown in colour, indicating that the removal of NOM was not due to adsorption to the biomass.

![Figure 5.10 History plots for *T. versicolor* cultures in Waksman medium (2 g/L glucose) containing 100 mg C/L NOM incubated at 30°C, inoculated with either spore suspensions or three agar plugs.](image)
To determine if there was adsorption of NOM to the agar component of the MEA plugs an experiment was carried out where three MEA plugs (each 1 cm²) without the fungus were added to 200 mL Waksman medium (2 g/L glucose) and sterilised NOM (final concentration of 100 mg C/L). The flask was then incubated at 30°C and 130 rpm for eight days.

The $A_{446}$ and $A_{254}$ stayed constant throughout the incubation period (Figure 5.11). This verified that there was no NOM adsorption to the agar, nor detectable leaching of brown-coloured material from the plugs.

![Figure 5.11](image)

**Figure 5.11** $A_{446}$ and $A_{254}$ for Waksman medium (2 g/L glucose) containing 100 mg C/L NOM and three agar plugs without fungus.

The total NOM removed (mg) in terms of both coloured and UV-absorbing species for cultures with plug inoculation was less, although a higher level of Lac activity was obtained (Figure 5.12). The ratio of $A_{254}$ to $A_{446}$ removal for the plug cultures was lower than for the spore cultures, 0.56 and 0.71, respectively.

Although the degree of NOM removal (measured as decolourisation) was 8% greater when the spore suspension inoculum was used, subsequent investigations were conducted using the plug inoculation method, as it was much simpler. Furthermore, plug inoculation led to higher enzyme activity than spore inoculation, even though there was a longer lag phase for the enzyme secretion, and thus provides better potential for the biodegradation of NOM.
Figure 5.12 NOM removals (as mg, converted from A_446 and A_254) and laccase activity in *T. versicolor* cultures, inoculated with either spore suspension or plugs.

5.1.4 Effect of NOM concentration

The effect of initial NOM concentration on NOM degradation was examined in order to determine the maximum concentration tolerated by the fungus. Cultures containing 200 mL Waksman medium (2 g/L glucose) and varying NOM concentration (final concentrations of 100, 300, 400, 500, 600 and 700 mg C/L) were prepared and each inoculated with three fungal plugs and incubated at 30°C and 130 rpm for ten days.

Figure 5.13 NOM removals (as mg, converted from A_446 and A_254), and glucose consumption in *T. versicolor* cultures with different NOM concentrations, plug inoculum.
NOM removed, when measured as both $A_{446}$ and $A_{254}$, increased with NOM concentration up to 600 mg C/L and decreased dramatically when the NOM concentration was increased to 700 mg C/L (Figure 5.13). The removal of NOM measured as colour ($A_{446}$) per unit glucose consumed increased linearly with NOM concentration up to 600 mg C/L NOM. However, the removal of UV-absorbing species per unit glucose consumed for 100-300 mg C/L NOM were approximately the same, increased linearly between 300-600 mg C/L and decreased considerably at 700 mg C/L NOM.

A linear relationship with good correlation ($R^2 = 0.9901$) was established between initial NOM concentration (100-600 mg C/L) and NOM removal in terms of $A_{446}$ (Figure 5.14). This relationship was in good agreement with the study by Rojek (2003), where the correlation found for *P. chrysosporium* in the NOM concentration range of 120-480 mg C/L was $y = 0.095x - 0.8521$ ($R^2 = 0.998$).

\[y = 0.0952x + 6.6653\]
\[R^2 = 0.9901\]

\[y = 0.0415x + 3.1796\]
\[R^2 = 0.9206\]

![Figure 5.14 NOM removals (as mg, converted from $A_{446}$ and $A_{254}$) by *T. versicolor* for different NOM concentrations.](image)

A linear correlation between initial NOM content (100-600 mg C/L) and NOM reduction in terms of $A_{254}$ was established: $y = 0.042x + 3.180$ ($R^2 = 0.9206$) (Figure 5.14). This differs from the observations of Rojek (2003), who reported that there was a similar extent of UV-absorbing NOM removed (approximately 15%) in all cultures and thus no relationship between $A_{254}$ and initial NOM content was established. However, a different NOM concentrate and fungus was used in that study, which may account for the different result. It
should be noted that both relationships obtained in the present study hold only for the NOM concentration range of 100-600 mg C/L.

The pattern of decolourisation and removal of UV-absorbing species increasing with initial NOM concentration can be correlated with the production of extracellular ligninolytic enzymes at different NOM contents (Figure 5.15). The Lac activity increased with NOM concentration up to 600 mg C/L, however, both LiP and MnP activities decreased with NOM content, indeed, there was no LiP activity when the NOM concentration exceeded 300 mg C/L. The culture with 700 mg C/L initial NOM content gave a markedly lower Lac activity. Dehorter and Blondeau (1992) illustrated a relationship between humic acid degradation and extracellular enzyme activity of *P. chrysosporium* and *T. versicolor*, and showed that the production of extracellular LiP and MnP increased with increasing concentrations of humic acids. The surfactant properties of the humic acids were suggested to be responsible for the increase in enzyme activities. However, 1% or higher concentrations of humic acids were found to be inhibitory to growth and enzyme induction. This is in an agreement with the present findings, where LiP and MnP activities decreased with NOM concentrations from 300 mg C/L (equivalent to 1.0%) to 700 mg C/L (equivalent to 2.3%).

![Figure 5.15](image.png)

**Figure 5.15** Activity of the extracellular phenoloxidase enzymes of *T. versicolor* in cultures containing varying NOM concentrations, plug inoculum.

As illustrated in Figure 5.14, NOM removal increased linearly with NOM concentration up to 600 mg C/L. This is coincident with the secretion of laccase, where the activity increased until 600 mg C/L NOM. The ratio of $A_{254}$ to $A_{446}$ for the 700 mg C/L culture was the lowest (0.23),
matching the sharp decrease in Lac activity. Thus, the removal of NOM from solution by *T. versicolor* was associated with high Lac activity. Again, the laccase in the cultures was responsible for breakdown of the NOM, probably by the cleavage of aromatic rings, conjugated and Cα-Cβ bonds in phenolic moieties, as well as catalysing alkyl-aryl cleavage.

### 5.2 Enhancement of Enzyme Production

According to the findings in Sections 5.1.1 and 5.1.4, it was established that laccase was the major extracellular enzyme secreted by *T. versicolor* and by deduction, involved in NOM removal. Consequently, the next phase of the study was the determination of the cultivation period at which maximum Lac activity occurred, and the effects of supplements, temperature and pH, and agitation.

Wheat bran was employed in this study, as it has been used to supply nutrients for white-rot fungal growth. Many researchers and industries have applied the solid-state fermentation (SSF) technique for the production of enzymes and spores by using lignocellulose residues (Ooijkaas *et al.* 2000, Pandey *et al.* 2000, Robinson *et al.* 2001, Couto & Sanromán 2005). There has been increased interest in SSF for the development of bioprocesses, such as bioremediation and biodegradation of hazardous compounds, biological detoxification of agro-industrial residues, biopulping, etc (Pandey *et al.* 2000) due to the recent improvements in reactor designs (Couto & Sanromán 2005).

Medium with higher glucose content (5 g/L) was utilised in this study as previous experiments showed that *T. versicolor* did not grow well at lower glucose concentration (2 g/L) without the presence of NOM. NOM was not added to the cultures since preliminary experiments demonstrated that NOM adsorbed to the wheat bran (Figure 5.16). There was a 15% decrease in $A_{446}$ although no fungus was cultured in the medium. To prevent adsorption of NOM to wheat bran, *T. versicolor* was pre-cultured in the wheat bran-containing Waksman medium in the absence of NOM to increase the production of ligninolytic enzymes.
Cultures were established in 250 mL Erlenmeyer flasks containing 100 mL of modified Waksman medium (5 g/L glucose) supplemented with 4.5 g/L wheat bran (Nyanhongo et al. 2002) or 0.5% (v/v) Tween 80 (Couto et al. 2001), or both, and three agar plugs (each 1 cm²) of T. versicolor. All fermentations were performed in duplicate.

### 5.2.1 Determination of cultivation time based on maximum laccase activity

The cultivation period at which maximum Lac activity occurred was determined. This time was then used for subsequent experiments involving enzyme assays and NOM removal.

The fungus was cultured in modified Waksman medium supplemented with 4.5 g/L wheat bran at 30°C and 130 rpm. Samples (3 mL) were collected periodically and centrifuged prior to pH, Lac activity and glucose measurements.

Microbial growth became apparent on day 4 and so the measurements were undertaken thereafter. The pH dropped markedly until day 7, after which it stayed constant. Lac activity became apparent from day 4 and peaked on day 9, the increase coinciding with the exhaustion of glucose on day 8 (Figure 5.17). Therefore the cultivation time for maximum Lac activity of T. versicolor under the conditions used was established as nine days.

**Figure 5.16** $A_{446}$ for 100 mg C/L NOM incubated in Waksman medium containing 4.5 g/L wheat bran in the absence of fungus.
5.2.2 Effect of supplements

Two supplements were employed in this study, viz., Tween 80 and wheat bran. Before inoculation with *T. versicolor* plugs, the culture medium without added NOM was supplemented with 0.5% (v/v) Tween 80 or 4.5 g/L wheat bran, or both, and was then incubated at 30°C and 130 rpm. After nine days (as established in the preceding experiment), the cultures were filtered through a fine sieve to remove the fungal plugs and pellets and the remaining wheat bran, and then were centrifuged at 8500 rpm (12,200 RCF) and 20°C for 15 minutes. The supernatant was frozen and then defrosted and the precipitated polysaccharides were removed by centrifugation (4400 rpm for 30 minutes) (Nyanhongo *et al.* 2002). The resulting clear solution was used for enzyme activity assays.

Table 5.1 indicates the activity of the extracellular phenoloxidase enzymes (LiP, MnP and Lac) of *T. versicolor* for the different culture conditions. There was little growth apparent in cultures in the absence of NOM and supplements; hence these displayed very little enzyme activity. Addition of wheat bran led to high Lac activity compared with LiP and MnP, demonstrating its great potential as a laccase inducer in submerged cultures of *T. versicolor*. This result is consistent with those reported by Lorenzo *et al.* (2002), who found that lignocellulosic materials can stimulate the laccase-producing ability of *T. versicolor*; this effect was attributed to the cellulose content of the residues. Furthermore, these residues act as a source of nutrients to the fungus for secondary metabolism, consequently they have been termed support-substrates. This suggests that wheat bran gives *T. versicolor* an environment...
similar to its natural habitat (wood), which would probably stimulate the fungus to secrete lignin-degrading enzymes.

Table 5.1  Comparative ligninolytic enzyme activities in different culture media.

<table>
<thead>
<tr>
<th>Culture broths</th>
<th>LiP</th>
<th>MnP</th>
<th>Lac</th>
</tr>
</thead>
<tbody>
<tr>
<td>No supplement</td>
<td>-</td>
<td>-</td>
<td>0.30</td>
</tr>
<tr>
<td>+ 0.5% Tween 80</td>
<td>-</td>
<td>-</td>
<td>27.0</td>
</tr>
<tr>
<td>+ 4.5 g/L wheat bran</td>
<td>0.65</td>
<td>7.84</td>
<td>60.3</td>
</tr>
<tr>
<td>+ 4.5 g/L wheat bran &amp; 0.5% Tween 80</td>
<td>1.83</td>
<td>13.8</td>
<td>955</td>
</tr>
</tbody>
</table>

Addition of Tween 80 alone to the cultures led to a small improvement in Lac activity, being similar to the effect of the presence of 100 mg C/L NOM (Figure 5.15). However, with the presence of wheat bran it caused 1.8-fold, 0.8-fold and 15-fold increases in LiP, MnP and Lac activities, respectively. The cultures with the additions of the two supplements would probably be able to break conjugated, Cβ-C4 and Cα-Cβ bonds and cleavage aromatic rings of NOM due to the presence of the three-ligninolytic enzymes, particularly laccase. Asther et al. (1987) suggested that Tween 80, a non-ionic surfactant, was capable of transforming the cell membrane structure to promote permeation of LiP from the cell into the medium. Another study reported that Tween 80 can protect LiP in culture broths against inactivation due to agitation (Venkatadri & Irvine 1990). These observations are consistent with those of Shim and Kawamoto (2002) where the addition of Tween 80 to a culture of *P. chrysosporium* immobilised on Biolace resulted in a slight increase in LiP activity.

In spite of the increases in the ligninolytic enzyme activities for cultures in the presence of the two supplements, the activities of LiP and MnP were still low compared with those for the cultures in the presence of 300 and 400 mg C/L NOM, respectively (Figure 5.15). However, the combination of the two supplements led to markedly greater activity of laccase compared with the NOM-containing cultures in the range 100-700 mg C/L. Consequently, wheat bran and Tween 80 can act synergistically in improving the production of laccase, but not MnP and LiP, compared with cultures in the presence of NOM.

Determinations of the optimal pH and temperature of laccase secreted by the cultures supplemented with wheat bran and wheat bran plus Tween 80 were investigated as described below.
5.2.3 Effect of temperature and pH on laccase activity

The effect of temperature on Lac activity was established by performing assays at temperatures ranging from 25°C to 90°C at pH 4.5. The effect of pH was determined by measuring the activity at pH varying from 2.5 to 7.0 at the determined optimum temperature established in the preceding experiments.

The assay mixtures were equilibrated at the different temperatures and pH for 2.5 minutes before introducing the substrate guaiacol. Lac activity was determined by measuring the initial velocity of reaction.

(i) Effect of temperature

The activity of the enzyme secreted in the presence of Tween 80 was higher over a broader temperature range (50-70°C compared with 60-70°C), but decreased rapidly at greater than 70°C compared with the enzyme secreted in the absence of the detergent (Figure 5.18). The culture in the presence of Tween 80 retained 91-100% Lac activity over the temperature range of 50-70°C, whereas 93-100% Lac activity was maintained over 60-70°C by the culture in the absence of the detergent.

![Figure 5.18](image)

Figure 5.18 Effect of temperature on Lac activity at pH 4.5 in cultures supplemented with 4.5 g/L wheat bran (+WB) and 4.5 g/L wheat bran plus 0.5% Tween 80 (+WB +Tw80). The data points correspond to mean values of duplicate assays.

50°C was chosen as the temperature for enzyme assays as the enzyme may not be able to tolerate the higher temperature if incubated for extended periods.
(ii) Effect of pH

As for temperature, there was a similar broadening of pH range close to maximum activity for enzyme secreted in the presence of 0.5% Tween 80, the activity at pH $\geq 5$ being markedly greater for the latter (Figure 5.19). The culture in the absence of Tween 80 retained 100% Lac activity over the pH range of 4-4.5, whereas 92-100% Lac activity was maintained over pH 4-5 by the culture in the presence of the detergent.

![Figure 5.19](image.png)

**Figure 5.19** Effect of pH on Lac activities at 50°C in cultures supplemented with 4.5 g/L wheat bran (WB) and 4.5 g/L wheat bran plus 0.5% Tween 80 (WB + Tw80). The data points correspond to mean values of duplicate assays.

The culture fluids were tested neat, and gave results as in Figure 5.18 and Figure 5.19. As the activity of the wheat bran plus Tween 80 preparation was markedly higher, it was diluted in buffer to approximately the same activity as the wheat bran preparation, and retested (Figure 5.20). Although the dilution was somewhat greater than anticipated on the basis of calculation, the results for the diluted enzyme preparation confirmed those for the original culture fluid, i.e., higher Lac activity over broader temperature (50-70°C cf. 60-70°C) and pH (4.0-5.0 cf. 4.0-4.5) ranges than the wheat bran preparation.
Figure 5.20 Effects of (A) temperature and (B) pH on Lac activities after dilution for cultures supplemented with 4.5 g/L wheat bran plus 0.5% Tween 80 (+WB +Tw80).

The optimum temperature for the Lac activity from the cultures with wheat bran and wheat bran plus Tween 80 was determined to be 50°C although the highest activities were obtained at 70°C (WB preparation) and 60°C (WB+Tw80 preparation) as the Lac activities would deteriorate at higher temperature. The optimum activity of the enzyme from the two cultures of *T. versicolor* was identified as pH 4.0-4.5. The results were consistent with the study by Coll *et al.* (1993), who stated that the optimum temperature and pH for Lac activity secreted by *T. versicolor* was 50°C and pH 4.0-5.0, respectively.

5.2.4 Effect of agitation

There are several forms of growth that may affect fungal performance, namely filaments, fungal mats and pellets. It was reported that stationary cultures formed fungal mats whereas agitation led to pellet formation (Swamy & Ramsay 1999). Agitation provides better aeration, which in turn may result in greater decolourisation, however, some researchers showed that agitation could suppress fungal phenoloxidase enzyme activity (Kirk *et al.* 1978, Kirk & Farrell 1987).

In this experiment, cultures with three plugs (each 1 cm²) of *T. versicolor* and different medium contents (Table 5.2) were prepared and two different incubation conditions were applied: (A) continuous agitation at 130 rpm and 30°C and (B) agitation every 6 hours for 30 minutes at 130 rpm and 30°C. Condition (B) was selected as reducing agitation may prevent damage to the mycelium and secreted enzymes caused by shear stress and/or changes in the
morphology of the fungus (Rancaoño et al. 2003). These conditions may provide a trade-off between providing good aeration and inhibition of fungal ligninolytic activity. All cultures were run as duplicates.

### Table 5.2 Different medium contents used for *T. versicolor* cultures for testing effect of agitation.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Culture content</th>
</tr>
</thead>
<tbody>
<tr>
<td>WM</td>
<td>Waksman medium (WM, 5.0 g/L glucose)</td>
</tr>
<tr>
<td>WM + Tw80</td>
<td>WM + 0.5% Tween 80</td>
</tr>
<tr>
<td>WM + WB</td>
<td>WM + 4.5 g/L wheat bran</td>
</tr>
<tr>
<td>WM + WB + Tw80</td>
<td>WM + 4.5 g/L wheat bran + 0.5% Tween 80</td>
</tr>
<tr>
<td>WM + NOM</td>
<td>WM + 500 mg C/L NOM</td>
</tr>
<tr>
<td>WM + NOM + Tw80</td>
<td>WM + 500 mg C/L NOM + 0.5% Tween 80</td>
</tr>
</tbody>
</table>

Cultures with continuous agitation (Figure 5.21A) consumed glucose faster than those agitated every 6 hours for 30 minutes (Figure 5.21B), except for the culture without supplement. The high glucose consumption rates were probably due to the improved mass transfer of oxygen and substrates in agitated cultures.

The cultures agitated every 6 hours formed fungal mats on the surface of the medium whereas continuous agitation led to pellet formation. The level of Lac activity increased with the addition of Tween 80 for both incubation conditions. Tween 80 appeared to have the ability to protect enzymes in the culture fluids against mechanical inactivation due to agitation, as reported by Jäger et al. (1985). In addition, it was reported that Tween 80 acts as a LiP inducer (Asther et al. 1987) and so may also be effective in stimulating other enzymes such as laccase. The WM and WM + Tw80 cultures which were agitated every 6 hours for 30 minutes showed increasing Lac activity with time unlike those under continuous agitation, as less agitation prevented damage to the mycelium and secreted enzymes caused by shear stress and/or changes in the morphology of the fungus. However, continuous agitation for the two cultures resulted in low increase rate of the enzyme activity with time after day 9, which can be attributed to shear stress.

For the cultures in the presence of wheat bran and wheat bran plus Tween 80, enzyme activity increased with time for occasional agitation whereas decreased sharply, probably due to denaturation, after day 9 for the continuously agitated cultures. Again, Tween 80 is able to
increase secretion of laccase and/or, protect laccase significantly from inactivation under agitated conditions as the Lac activity remained high in both conditions with the addition of the detergent in the presence of wheat bran, especially for the cultures with continuous agitation.

![Graph A](image1)

![Graph B](image2)

**Figure 5.21** Glucose consumption (g/L) and Lac activity (U/L) for *T. versicolor* cultures in different media and agitation conditions: (A) continuous agitation and (B) agitated every 6 hours for 30 minutes, both at 30°C and 130 rpm.

Lac activity in the cultures provided with NOM and NOM plus Tween 80 increased with time for both conditions. Addition of NOM enhances Lac activity as observed in Section 5.1.4 (Figure 5.15) where laccase increases with NOM concentration up to 600 mg C/L. However, there was no increase in laccase in the presence of Tween 80, indeed, there was a decrease, in contrast with the induction of laccase by the combination of wheat bran and Tween 80. Thus, the addition of wheat bran or NOM alone, and the combination of wheat bran plus Tween 80
can stimulate the laccase-producing ability of *T. versicolor*, however, addition of Tween 80 alone did not give significant improvement.

Fermentations with continuous agitation promote higher and faster enzyme activity than those with occasional agitation because it provides better mass transfer. This is consistent with the findings of Knapp *et al.* (1997), who demonstrated that agitation was very effective in improving the rate of Orange II decolourisation by mycelial pellets of an unidentified basidiomycete fungus. This may be due to the improved mass transfer of oxygen and substrates in agitated cultures. However, it seemed that continuous agitation had an adverse effect on the fungal growth and enzyme activity over longer fermentation periods. Thus discontinuous agitation like condition (B) would be a better alternative if longer contact time were required.

![Graph showing absorbance at 446 nm and 254 nm](image)

**Figure 5.22** $A_{446}$ and $A_{254}$ for cultures in the presence of 500 mg C/L NOM, and 500 mg C/L NOM plus Tween 80, agitated continuously at 30°C and 130 rpm.

Removal of NOM in terms of $A_{446}$ and $A_{254}$ was slightly higher for cultures with NOM alone than those with NOM plus Tween 80 (Figure 5.22). This is consistent with the laccase results, where the culture with NOM only has a higher laccase level than the culture with Tween 80, further evidence for the role of laccase in the breakage of bonds that lead to reduction in $A_{446}$ and $A_{254}$. *T. versicolor* removed 86% and 82% (measured as $A_{446}$) and 43% and 39% (measured as $A_{254}$) of the NOM from the NOM and NOM + Tw80 cultures, respectively.
5.3 Enzymatic Treatment of NOM

Filtered extracellular culture fluid obtained from the WB + Tw80 culture at 9 days was incubated with NOM and NOM removal at varying pH and temperature was investigated. The reaction mixture contained 4.0 mL of the culture fluid, 3.0 mL of Na$_2$HPO$_4$-citric acid buffer and 3.0 mL of NOM (final concentration of 300 mg/L). The controls (NOM + buffer + water) showed insignificant changes in $A_{446}$ and $A_{254}$ at different pHs and temperatures.

5.3.1 Effect of pH

The reaction mixtures were prepared with buffers in the range pH 3-7. The mixture was incubated in a water bath set at 30°C. The Na$_2$HPO$_4$-citric acid buffer formulations are tabulated in Table 5.3.

<table>
<thead>
<tr>
<th>pH at 18°C</th>
<th>0.2M Na$_2$HPO$_4$ (mL)</th>
<th>0.1M citric acid (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>0.855</td>
<td>9.145</td>
</tr>
<tr>
<td>3.0</td>
<td>2.055</td>
<td>7.945</td>
</tr>
<tr>
<td>4.0</td>
<td>3.855</td>
<td>6.145</td>
</tr>
<tr>
<td>5.0</td>
<td>5.150</td>
<td>4.850</td>
</tr>
<tr>
<td>6.0</td>
<td>6.315</td>
<td>3.685</td>
</tr>
<tr>
<td>7.0</td>
<td>8.235</td>
<td>1.765</td>
</tr>
</tbody>
</table>

There was reduction in $A_{446}$ and $A_{254}$ for all pHs except pH 7 for which there was an increase in $A_{254}$ (Figure 5.23). The increase in $A_{254}$ at pH 7 was probably due to a change in the NOM structure. The NOM removal ability for *T. versicolor* measured as $A_{446}$ and $A_{254}$ improved with pH up to 4.5, above which it decreased markedly with increasing pH. The ratios of $A_{254}/A_{446}$ for pH 3-5 were ~0.77, whereas they were ~0.58 for pH 6 and 7. The different ratios of $A_{254}/A_{446}$ suggest that different bonds were broken by laccase at the different pH. The trend for NOM removal matched the results for optimal pH for Lac activity, pH 4.5 (Figure 5.19); hence the optimum pH for the reaction was determined as pH 4.5.

The results agree with those of many researchers (Zhang *et al.* 1999, Kapdan *et al.* 2000, Aktas & Tanyolac 2003, Rancaño *et al.* 2003), in that the optimum pH for laccase was pH 4-5, even though different substrates were utilised. Kapdan *et al.* (2000) stated that the dyestuff decolourisation efficiencies of *C. versicolor* MUCL were lower at pH 6 and 7 compared with
pH 4.5, which is consistent with the current results. However, one research group found that the optimum pH for the enzymatic decolourisation of Remazol Brilliant Blue R (RBBR) by Funalia trogii ATCC 200800 growing in a solid-state fermentation (SSF) medium containing wheat bran and soybean waste was pH 3.0 (Deveci et al. 2004), contrary to the present results.

Figure 5.23 Reduction in $A_{446}$ and $A_{254}$ at different pH.

5.3.2 Effect of temperature

The reaction mixtures were prepared at pH 4.5 since it was the optimum value found in the preceding experiments. The mixtures were incubated in water baths at different temperatures in the range 30-80°C.

The optimum temperature for the reaction was established as 50°C (Figure 5.24), i.e., at which maximum reduction in $A_{446}$ and $A_{254}$ occurred. The ratios of $A_{254}/A_{446}$ for the temperature range 30-50°C were 0.77-0.79, whereas they were 0.58-0.69 for 60-80°C. As with pH, different ratios of $A_{254}/A_{446}$ at the different temperatures were obtained, suggesting that different bonds are being broken by laccase at the different temperatures. There was a comparable reduction in $A_{254}$ at 30-50°C, less reduction at 60°C and an increase at 70-80°C, the latter probably due to thermal disruption of the NOM structure. No reduction in $A_{446}$ was observed for cultures at 70-80°C. These observations were different from when guaiacol was used as a substrate to investigate the effects of temperature on Lac activity (Figure 5.18) where high Lac activity occurred at 50-70°C.
Figure 5.24  Reduction in $A_{446}$ and $A_{254}$ at different temperatures.

Therefore, the effect of temperature varies when different substrates are used. Schliephake et al. (2000) reported that the laccase from *Pycnoporus cinnabarinus* CBS 101046 when used for the degradation of the diazo dye Chicago Sky Blue was stable at 60°C for one hour only, but remained active at 37°C for long periods (25 days). Nyanhongo et al. (2002) showed that different dyes were decolourised at different rates at different temperatures for which Lac activity increased with temperature up to 50-60°C, after which it decreased. Although the optimum temperature for laccase when using guaiacol as a substrate was 50°C, it was not so clear-cut for NOM. Hence, 30°C was chosen as the incubation temperature for the *in vitro* studies of Lac activity, as the laccase may be denatured if the NOM removal process were run for long period at high temperature (30°C cf. 50°C), and it corresponds to the temperature at which fungal growth (Figure 5.2) and enzyme production (Figure 5.4) were high (30°C cf. 36°C).
5.4 Biodegradation of NOM by *T. versicolor*

As discussed in Section 5.2.2, culture medium without added NOM supplemented with Tween 80 or wheat bran, or both, produced low LiP and MnP activities but high Lac activity compared with the cultures in the presence of NOM. This led to a further study to investigate the potential of NOM as an inducer of the phenoloxidase enzymes in the presence of the two supplements. Although a preliminary experiment (results shown in Figure 5.16) illustrated that adsorption of NOM to wheat bran occurred, it was not to a great extent, and so experiments were conducted to investigate the trade-off between adsorption and enzyme induction, and hence NOM degradation.

Cultures containing 200 mL Waksman medium (5 g/L glucose) supplemented with 4.5 g/L wheat bran and 0.5% (v/v) Tween 80, and sterilised NOM (final concentration of 100, 600 and 700 mg C/L) were prepared and each was inoculated with three fungal plugs. The cultures were incubated at 30°C and 130 rpm for thirteen days. All fermentations were performed in duplicate.

![Figure 5.25](image)

**Figure 5.25** History plot for *T. versicolor* cultures containing 100 mg C/L NOM, supplemented with 4.5 g/L wheat bran and 0.5% (v/v) Tween 80, Waksman medium with 5 g/L initial glucose, incubated at 30°C and 130 rpm.

Unlike the history plot for *T. versicolor* culture with no added supplements incubated at 30°C (Figure 5.1), the pH drop occurred over the first five days, after which the pH increased slightly (Figure 5.25). The pH fall coincided with increase in glucose consumption, but the A$_{446}$ and A$_{254}$ did not vary greatly. The major reductions in the absorbances happened only
after day 5. It should be noted that the apparent glucose consumption was greater than that provided (5 g/L) as the wheat bran also provided some reducing sugar as a carbon source for the fungus.

The slow decreases in both $A_{446}$ and $A_{254}$ within the first five days, similar to the trends for the cultures with no supplement, suggested that there was no or very little adsorption of NOM on the fungal pellets or wheat bran despite the pH drop, which can lead to adsorption. There was probably enzyme induction during the period and thus breakdown of NOM when there were marked reductions in $A_{446}$ and $A_{254}$ from day 5-13. High removals of colour and UV-absorbing compounds were achieved, 79% and 49% respectively. However, it should be noted that the initial $A_{446}$ and $A_{254}$ for these systems were higher than the cultures with no supplement due to the added coloured and UV-absorbing materials from the wheat bran. Hence, the cultures with 100, 600 and 700 mg C/L NOM in the presence of wheat bran and Tween 80 contained 26, 128 and 148 mg total carbon contents (converted from $A_{446}$), respectively. As there was 5.0-5.6 mg of the colour added to the culture fluids due to the wheat bran, a corrected removal of NOM must be considered.

![Figure 5.26](image)

Table 5.26 NOM removals (as mg, converted from $A_{446}$ and $A_{254}$), and glucose consumption in *T. versicolor* cultures with the two supplements and different NOM concentrations, plug inoculum.

The overall apparent reductions in colour for the 100, 600 and 700 mg C/L NOM cultures were 79%, 61% and 17%, respectively (Figure 5.26). The highest apparent removal of NOM measured as $A_{446}$ and $A_{254}$ was obtained for the 600 mg C/L NOM cultures. The 700 mg C/L
NOM cultures had higher and lower apparent reductions in colour and UV-absorbing molecules, respectively, than the 100 mg C/L NOM cultures. These trends matched the trends for the NOM cultures without any supplements (Figure 5.13). The NOM removed per unit glucose consumed followed the same trends, where the 600 mg C/L cultures had the highest.

When taking the colour from the wheat bran into account, the colour reductions for the cultures with 100, 600 and 700 mg C/L NOM were 73%, 60% and 14%, respectively. No improvement in NOM removal was obtained for all the cultures with the addition of supplements, except that the culture containing 600 mg C/L NOM had 11 mg more reduction in colour.

The adsorption of NOM on the wheat bran must be considered. To calculate the amount of NOM adsorbed to the wheat bran, adsorption experiments were set up for three different NOM concentrations (100, 600 and 700 mg C/L) in the absence of T. versicolor (Figure 5.27).

![Figure 5.27 A<sub>446</sub> for 100, 600 and 700 mg C/L NOM incubated in Waksman medium (5 g/L glucose) containing 4.5 g/L wheat bran in the absence of fungus, as controls.](image)

There was approximately 16% adsorption of NOM by the wheat bran for all the cultures with different NOM concentrations. Therefore, the overall decolourisations after colour and adsorption corrections due to the addition of wheat bran were 61%, 50% and 12% for the cultures containing 100, 600 and 700 mg C/L NOM, respectively. After all, there was no improvement in colour removal with the addition of the two supplements for all the cultures compared with those in the absence of wheat bran and Tween 80.
The marked increases in LiP and MnP activities but decrease in Lac activity led to no improvement in NOM removals for the systems (Figure 5.28). There was no increase in NOM removal compared with the cultures with no supplements, probably due to the very large drop in Lac activity. This is further evidence that laccase played a major role in the removal of NOM.

![Graph showing enzyme activity (U/L) vs. Initial NOM concentration (mg C/L)](image)

**Figure 5.28 Activity of the extracellular ligninolytic enzymes of *T. versicolor* in cultures containing the two supplements and different NOM concentrations, plug inoculum.**

As established in Section 5.2.2, addition of the wheat bran and Tween 80 combination enhanced LiP, MnP and Lac production. However, the LiP and MnP activities were low in the absence of NOM (Table 5.1). By adding NOM to the cultures supplemented with both wheat bran and the detergent, Lac activity decreased markedly. In contrast, the addition of wheat bran and Tween 80 to the NOM cultures led to increases in LiP and MnP activities, especially LiP. The enhancement of LiP and MnP activities was attributed to the protective ability of Tween 80 against mechanical inactivation of the enzymes (Venkatadri & Irvine 1990), and subsequently led to a greater NOM removal. The results are consistent with the findings of Venkatadri and Irvine (1990), who reported that the presence of Tween 80 caused a 1.3- to 1.4-fold increase in LiP enzyme activity. Furthermore, Tween 80 can aid the secretion of LiP by promoting the permeation of LiP from the cell into the medium (Asther *et al.* 1987). Therefore, the combination of NOM, wheat bran and Tween 80 can lead to increases in LiP and MnP activities, however, they had an adverse effect on laccase although wheat bran and Tween 80 can induce the production of laccase.
The overall apparent removal of NOM for the supplemented system with 100 mg C/L NOM was 79%, however, the colour reduction was corrected to 73% due the colour from the wheat bran. The NOM removal after corrected for colour from and adsorption by wheat bran was markedly reduced to 61%. The addition of wheat bran and Tween 80 to cultures of *T. versicolor* did not improve the decolourisation/removal of NOM for all the cultures when adsorption by and colour from the wheat bran were taken into account.
Chapter 6 Conclusions and Recommendations

6.1 Conclusions

*Phanerochaete chrysosporium* ATCC 34541 had been shown to remove 40-50% NOM from solution largely by adsorption rather than biodegradation (Rojek 2003). Consequently, the aim of this research was to develop an improved biological treatment for the bioremediation of NOM wastes.

When cultured in the presence of NOM, *P. chrysosporium* caused NOM removal and decolourisation largely by adsorption. *P. chrysosporium* seemed to preferentially remove the VHA fraction of the NOM, and so was most effective for the NOM preparation with the highest VHA content (i.e., greatest colour, aromaticity and the highest SUVA). *P. chrysosporium* cultures grown in media with different C:N ratios achieved the greatest decolourisation with the lowest the C:N ratio: Waksman (C:N = 6) > Fahy (C:N = 76) > Fujita medium (C:N = 114). This is in agreement with the findings of Rojek (2003), who also demonstrated that the removal of NOM increased with decreased C:N ratio (1.58-15.81) for a different NOM preparation.

Of the organisms tested, the two strains of *P. chrysosporium* (ATCC 34541 and ATCC 24725) and *Saccharomyces* spp. 1-3 removed NOM primarily by adsorption as indicated by the deep brown colouration of the biomass, whereas *T. versicolor* had the greater removals (59%) and the NOM removal was largely due to biodegradation.

The higher NOM removal with *T. versicolor* at 30°C corresponded to the greater production of the oxidative enzymes than at 36°C (73% vs. 50%), suggesting reduced production of the phenoloxidase enzyme activity at higher temperature. Increasing initial glucose content from 2 to 5 g/L did not improve NOM removal (48% cf. 73%), and biosorption occurred due to the uptake of the NOM by the fungal cell walls and low enzyme activities were present. Increasing NOM concentration led to increasing NOM removal and different activities of the ligninolytic enzymes. The increase in NOM removal coincided with increasing Lac activity but decreasing LiP and MnP activities, indicating laccase played a major role in NOM degradation.

Addition of wheat bran, and wheat bran plus Tween 80, to the cultures caused increases in the activities of LiP, MnP and Lac, especially laccase, as wheat bran can enhance the production
of the extracellular phenoloxidase enzymes (Couto et al. 2001, Lorenzo et al. 2002) whereas Tween 80 can protect LiP against mechanical inactivation due to agitation as well as act as an inducer (Asther et al. 1987, Venkatadri & Irvine 1990). Although the optimum activity of the enzyme from the culture supplemented with 4.5 g/L wheat bran plus 0.5% Tween 80 occurred at 50°C, 30°C was recommended as the ligninolytic enzyme activity may deteriorate if the NOM removal process were run for long period at high temperature. The optimum pH for the enzyme was identified as pH 4.0-4.5.

Although agitation led to apparent enzyme denaturation, fermentations with continuous agitation promote faster and higher enzyme activity than those with occasional agitation due to better mass transfer conditions. However, it seemed that continuous agitation had an adverse effect on the fungal growth and enzyme production over longer fermentation periods (greater than 9-13 days). Thus, discontinuous agitation would be a better option if extended fermentation were required.

In the wheat bran and Tween 80 systems, there were two corrections needed to be taken into account: colour from wheat bran, which contributed to the overall apparent NOM colour, and adsorption of NOM on to the wheat bran. Addition of NOM to cultures of *T. versicolor* supplemented with wheat bran and Tween 80 led to markedly reduced Lac activity, but increased LiP and MnP activities, and no enhancement in NOM removal compared with the cultures in the absence of supplements (12 mg (or 61%) cf. 15 mg (or 73%) for 100 mg C/L after corrected for colour from and adsorption by wheat bran).

### 6.2 Recommendations

As the biological treatment of NOM wastes was conducted in shake flasks, further investigations in a bioreactor would be recommended to determine the optimal operational conditions such as dissolved oxygen levels and agitation. Kirk et al. (1978) suggested that the lignin-degrading system required high O₂ demand and so the impact of dissolved oxygen on NOM removal may play a role in the biological treatment. Furthermore, Knapp et al. (1997) demonstrated that agitation was very effective in improving (more than doubling) the rate of Orange II decolourisation by mycelial pellets of an unidentified basidiomycete fungus due to the improved mass transfer of oxygen and substrates in agitated cultures. Agitation may therefore influence the performance of NOM removal and could be optimised via a trade-off between enhanced mass transfer and ligninolytic enzyme denaturation. The operational
conditions could easily be optimised as the control of culture conditions can more easily be managed in bioreactors.

NOM removal by *T. versicolor* immobilised in a membrane bioreactor or on other suitable substrate may be an option to develop a reliable system applicable to drinking or wastewater treatment. There are two types of configurations of membrane bioreactor systems, submerged and side-stream (Mallia & Till 2001). Millia and Till (2001) reported that membrane bioreactor technology is able to produce consistently high effluent quality and to reduce disinfection as membranes with pore openings generally in the 0.1-0.5 mm range can trap a significant proportion of pathogenic organisms. Furthermore, membrane bioreactor reduces sludge production and requires a smaller design footprint. Shim and Kawamoto (2002) established that immobilisation of mycelia cell culture on a bio-carrier is more effective in promoting cell growth and LiP production compared to conventional stationary liquid culture.

The research was mainly focussed on the biological treatment of MIEX concentrate, further investigations could be done on other NOM wastes such as sludges from membrane plants and alum precipitation.
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Appendix 1  Correlation between NOM concentration and $A_{446}$ and $A_{254}$

Figure A1  The correlation between NOM concentrations and $A_{446}$ and $A_{254}$ for the three NOM preparations.
Appendix 2  Absorbance correction factor

Figure A2  pH impact on the absorbance at 446 nm and 254 nm.
The experiments were conducted twice and the samples for each were analysed three times. The mean value values were plotted.

The description of the calculation of corrected absorbance based on the plots on previous page (Figure A2) is explained in Table A2.

Table A2 Calculation of corrected absorbance.

<table>
<thead>
<tr>
<th>Y-axis</th>
<th>X-axis</th>
<th>Corrected absorbance, $A_{ci}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$CF(%) = \frac{A_{cl} - A_i}{A_i} \times 100$</td>
<td>$pH$ difference $= pH_0 - pH_i$</td>
<td>$A_{ci} = A_i + \frac{A_i \times CF}{100}$</td>
</tr>
<tr>
<td>$CF = $ Correction factor (%)</td>
<td>$pH_0 = $ Initial pH</td>
<td>$A_{ci} = $ Corrected absorbance on day $i$</td>
</tr>
<tr>
<td>$A_i = $ Measured absorbance on day $i$</td>
<td>$pH_i = $ pH on day $i$</td>
<td></td>
</tr>
<tr>
<td>$A_{ci} = $ Corrected absorbance on day $i$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix 3  Typical standard curve for glucose determination

Figure A3  Typical standard curve for glucose determination by DNS method.
Appendix 4   Standard curve for determination of apparent molecular weight (Dalton) in HPSEC analysis

log (Mw) = -0.399(t) + 7.205
R² = 1.000

Figure A4   Standard curve for HPSEC using polystyrene sulphonate standards.