# AN INVESTIGATION INTO THE BIOREMEDIATION OF BLACK OLIVE BRINE WASTEWATER

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## ABSTRACT

In South Africa, the table olive industry is burgeoning and as a consequence, the large volumes of fermentation wastewaters that are generated have created environmental concern, as these wastewaters have the potential to pollute rivers and groundwaters. Currently, these wastewaters are disposed of in large evaporation ponds, but this is not considered to be the optimal treatment solution, due to the potential for pollution and environmental damage. This thesis describes an investigation into the bioremediation of black olive fermentation wastewaters.

Wastewaters, from both the table olive and olive oil industries, are toxic and this toxicity can be attributed to the phenolic compounds present. These compounds are known to have antimicrobial and phytotoxic effects. Aerobic biological treatments have been extensively investigated in order to reduce the phenolic fraction of olive mill wastewaters (OMW) with relative success. Biological treatment methods are also cheaper than chemical or physical treatment methods. Therefore, it was decided to investigate aerobic biodegradation of black olive fermentation wastewater (olive wastewater) from the production of black (Kalamata) olives.

Isolated strains from the evaporation ponds of a nearby olive production plant were screened for their ability to grow in, and reduce, the total phenolic fraction of black olive fermentation wastewaters. The strain isolation was conducted using samples from the evaporation ponds because microbes found here would have the ability to metabolise the compounds present in the wastewater and would be acclimatised to the harsh, toxic environment posed by the wastewater. From the strains isolated, one (AS-35) was selected. This strain showed the greatest sequence homology with *Bacillus megaterium* and was then used in a comparative study with a commercially available white-rot fungus, *Trametes pubescens*, to determine which of the organisms had the best biodegradative capabilities. White-rot fungi produce extracellular enzymes, such as laccase, that enable them to break down lignin in wood. Since the components of olive wastewater are structurally similar to lignin, it was decided to investigate the ability of a

white-rot fungus, capable of producing high titres of laccase, for the biodegradation of olive wastewater. From these investigations, *T. pubescens* was found to have a greater capacity for reducing the phenolic fraction of olive wastewater and showed an ability to decolourise the wastewater not shown by the bacterial strain. For this reason, further studies were conducted with *T. pubescens* to determine the relationships between growth, extracellular laccase production and biodegradation of olive wastewater.

In these investigations, *T. pubescens* was able to reduce the total phenolic content of 10% and 25% (v/v) dilutions of olive wastewater by 82 and 84% respectively with concomitant COD reductions of 53 and 54% respectively. Biomass concentrations reached 2.44 and 5.39 g/L in 10% and 25% (v/v) olive wastewater, respectively. In parallel with these excellent biodegradation results was the presence of significant laccase activity. Therefore, an investigation was conducted on the bioremediation of olive wastewater with free laccase isolated from cultures of *T. pubescens*.

With the addition of free laccase (0.4 and 1 U/mL) to 5 ml samples of olive wastewater, total phenols were reduced by over 50% after 6 hours in dilutions up to 75% (v/v) and up to 40% in undiluted olive wastewater. After 20 hours of reaction, phenolic reduction was only marginally higher with the highest reduction (60%) occurring in the 50% (v/v) dilution of wastewater. Phenolic reduction was not improved by increased enzyme addition. It is proposed that the reduced efficiency observed with increased enzyme is related to deactivation of the enzyme. The oxidation products generated by the action of laccase may react irreversibly with the active site. With increased amounts of enzyme the rate of production of these oxidation products increased and may have resulted in an increased deactivation of excess enzyme. The reaction of laccase with components of the olive wastewater may also have been substrate limited and therefore increased amounts of enzyme had little effect. Deactivation of the enzyme appears to be a more plausible explanation, considering the findings of other researchers.

This study illustrated the ability of *T. pubescens* to reduce the toxicity of black olive fermentation wastewaters and has shown it to be an excellent candidate organism for

aerobic biodegradation of this wastewater. In addition, the studies with cell-free laccase showed high levels of phenol removal with a significantly shorter treatment time. It was calculated, from the data obtained, that in a 10% (v/v) dilution of olive wastewater, 1g of biomass could convert 0.24g phenols in 24 hours. On the other hand, 1g of biomass produced 369 U of laccase which could convert 111g phenols in undiluted olive wastewater, in a comparable amount of time. In effect, 462.5g of biomass would be required to convert the same amount (111g) of phenols as achieved with enzyme treatment. Laccase can be produced inexpensively in an olive wastewater medium and the treatment does not require dilution of the wastewater, but does require further downstream processing to isolate the enzyme. Laccase treatment may prove to be a more economically viable option for a full scale treatment process for the bioremediation of olive wastewater.

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# CHAPTER 1

# LITERATURE REVIEW

## 1.1 Introduction

The olive oil and table olive industry is one of the major agro-industrial activities in the Mediterranean region and is expanding in the Western Cape. The world production of table olives amounted to 1 456 000 tons in the 2001-2002 season. The black olive industry generates 0.9 – 1.9 $m^3$ of wastewater per ton of olives (Kopsidas, 1992). These wastewaters have a high organic load and pose an environmental hazard due to the phenolic compounds present. These phenolic compounds have antimicrobial and phytotoxic effects which prohibit their disposal into rivers and other waterways. Traditional methods of treatment, such as evaporation ponds, have the potential to pollute groundwaters and are not an optimal treatment solution. Disposal of the wastewaters into municipal water systems and rivers is illegal and has the potential problem of corroding sewer pipes due to the presence of organic acids. Therefore, an effective treatment method must be developed to treat this agro-industrial waste. Biotechnology is a powerful technology which harnesses the abilities of microorganisms to carry out certain biological processes. Biotechnological processes have the potential to produce economically and socially valuable products and also offer the potential to eliminate toxic compounds present in industrial wastes by biological conversions *via* enzymatic systems. Bioremediation has a negligible impact on the environment and it is less expensive than other physical or chemical treatment processes (Hafidi *et al.*, 2005).

In this study, an investigation to develop the technology for the bioremediation of black olive fermentation wastewater was undertaken. The aim of the study was to detoxify the wastewater using microorganisms isolated from olive waste-polluted sites. The efficiency of these was compared with that of a commercially available white-rot fungal strain. A white-rot fungus was investigated as these organisms are known for their ability to degrade aromatic pollutants with the use of extracellular enzymes.

## 1.2 Olive and olive oil production

Olive oil is produced in one of two ways: the traditional, discontinuous press method or the more modern, continuous centrifugation of a mixture of milled olives and hot water. The olives are destalked and rinsed and then crushed and mixed with hot water in a process known as malaxation. This allows for oil droplets to coalesce and thus increases the yield of oil. This mixture is centrifuged continuously, and depending on the type of system, either two or three phases are produced. In the two phase system, the pulp and vegetation waters form one stream while the olive oil constitutes the second stream. In the three phase system the vegetation waters and pulp emerge in two separate streams. The mixture of these vegetation waters and the washing waters used at different stages in the process constitutes olive mill wastewater (OMW). It has a dark red to black colour, which can be attributed to the high quantity of polyphenolic compounds (Sayadi *et al.,* 2000). The simple phenolic compounds pose an ecological hazard and are responsible for the antimicrobial and phytotoxic effects of these wastewaters that have been widely reported (Capasso *et al.,* 1992; Ramos-Cormenzana *et al.,* 1996; Rodriquez *et al.,* 1988; Borja *et al.,* 1990). There are several mechanisms of action that make these compounds antimicrobial, such as destabilization of cytoplasmic membranes, permeabilisation of plasma membranes, inhibition of extracellular enzymes and direct actions on microbial metabolism (Puupponen-Pimia *et al.,* 2005).

Table olives are of either green or black variety. There are three main industrial preparations of table olives, namely, green or Spanish style, black ripe or Californian-style, and naturally black or Greek-style (Kalamata) olives (Romero *et al.,* 2004). For each preparation, olives are harvested at different stages of fruit maturity. Spanish style olives are harvested when they are still green and naturally black olives are harvested at full maturity. The Californian olives are harvested in between these two stages (Romero *et al.,* 2004). The processes for black and green olive production are different (Fig. 1.1) and there are also many variations to these basic processes. The wastewaters generated during table olive production include the rinsing waters from washing the olives, the debittering waters from the lye treatment and the fermentation brines (OFW).

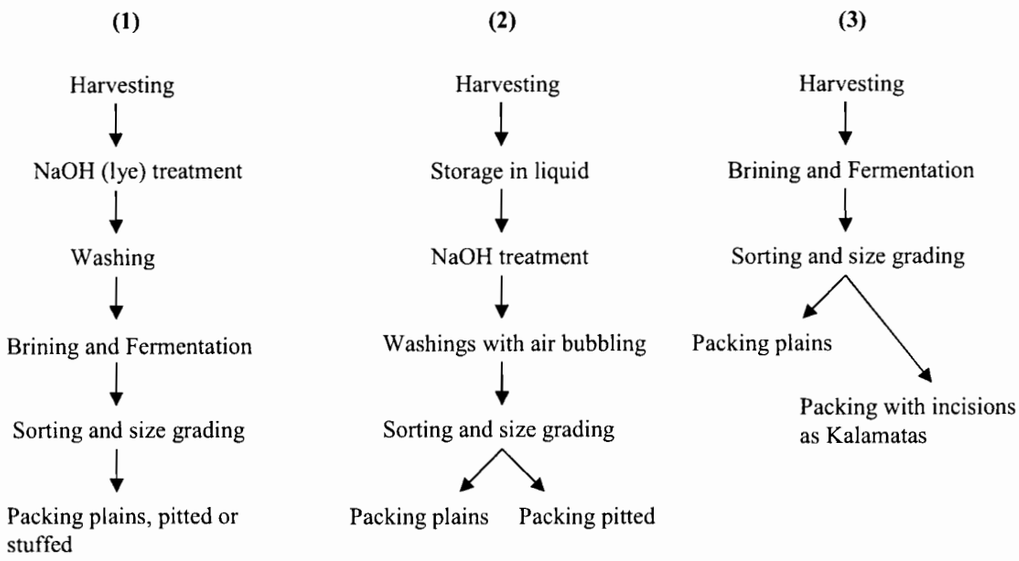



**Figure 1.1: Flow diagram representation for the processing of table olives: (1) Spanish-style green olives, (2) Black ripe olives, (3) Naturally black olives (Romero *et al.*, 2004).**



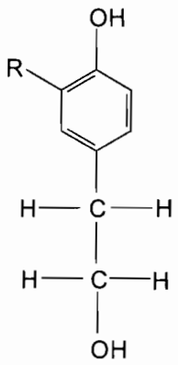
### 1.3 Composition of olive wastewaters

OMW and OFW have high organic loads and significant amounts of phenolic compounds, but they differ in composition. The compositions of OFW, especially that of black olives, have been poorly studied (Beltran-Heredia *et al.*, 2000; Benitez *et al.*, 2001). However, the composition of OMW has been well researched (Balice and Cera, 1984; Hamdi *et al.*, 1991; Borja *et al.*, 1992; Borja and Gonzalez, 1994; Borja *et al.*, 1995; Hamdi, 1996; Ramos-Cormenzana *et al.*, 1996; Rozzi and Malpei, 1996). Both have a high polluting organic load, expressed as Chemical Oxygen Demand (COD) or Biological Oxygen Demand (BOD). OMW has a pH range of 4 to 6, approximately 15% organic matter, including sugars (1 – 8%), nitrogen compounds (0.5 – 2.4%), organic acids (0.5 – 1.5%), fats (0.02 – 1%), as well as phenols and pectins (1 – 1.5%). The presence of various flavonoids, organic phenolic and non-phenolic acids and several minerals (K, Ca, Na salts) are also common (Moreno *et al.*, 1987).

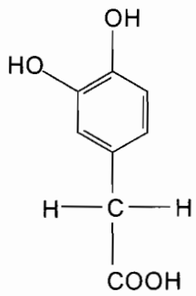
The phenolic compounds in OMW and OFW are either simple monophenols or complex polyphenols, tannins, pigmented anthocyanins and glucosides (Ramos-Cormenzana *et al.*, 1995; Greco *et al.*, 1999; Hamdi and Ellouz, 1992; Hamdi 1993). They can be broadly categorised as low, medium or high molecular weight compounds depending on their degree of polymerisation.

Hydroxytyrosol (3,4-dihydroxyphenylethanol) (Fig. 1.2) has been reported as the main phenolic compound in OMW (Romero *et al.,* 2002), originating from the hydrolysis of oleuropein, a glucoside ester of hydroxytyrosol and elenolic acid. Oleuropein is the compound found in the flesh of the olive and is characterised by the bitter taste (de Castro and Brenes, 2001) and its concentration declines as olives mature (Amiot *et al.,* 1986) due to its hydrolysis to hydroxytyrosol. Thus, black olive processing does not require the use of lye treatments and therefore these wastewaters are consistently acidic, with a high concentration of hydroxytyrosol.

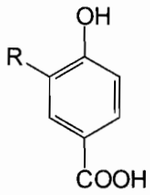
Other compounds reported to be present in olive wastewaters include tyrosol (4-hydroxyphenylethanol), 3,4-dihydroxyphenylacetic acid, 4-hydroxybenzoic acid, catechin, vanillic acid, vanillin, caffeic acid and *p*-coumaric acid (Fig. 1.2) (de Castro and Brenes, 2001; Brenes *et al.,* 1990). Reports of wastewater composition vary with regard to phenolic compounds. Rodriquez *et al.* (1988) reported that syringic and caffeic acids were not detected, while a study by Balice and Cera (1984) showed that these acids were observed in significant quantities.



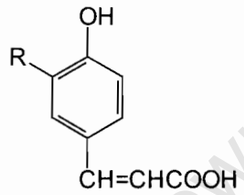

R = H Tyrosol
R = OH Hydroxytyrosol



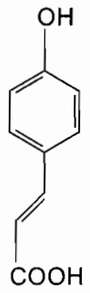

3,4-dihydroxyphenylacetic acid



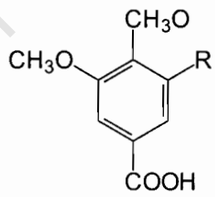

R = H 4-Hydroxybenzoic acid
R = OH 3,4-Dihydroxybenzoic acid
R = $OCH_3$ Vanillic acid




R = H Coumaric acid
R = $OCH_3$ Ferulic acid




4-Hydroxycinnamic acid




R = H 3,4-Dimethoxybenzoic acid
R = $OCH_3$ 3,4,5-Trimethoxybenzoic acid



**Figure 1.2: The predominant simple phenolic compounds identified in olive wastewaters (Balice and Cera, 1984; de Castro and Brenes, 2001 and Kyriacou *et al.*, 2005).**

The salinity of OFW is high (10 – 15%) and the sugar content is low as sugars are readily utilised during the brining of the olives. The composition is dependent on olive maturity and the process. OFW is characterised as having a suspended solids content of 0.03 – 0.4 g/L, dissolved solids of 0.2 – 80 g/L, Chemical Oxygen Demand (COD) of 0.3 – 60 g/L and a salt content of up to 150 g/L (Kyriacou *et al.,* 2005).

Hydroxytyrosol is abundant (up to 1g/L) in black olive wastewaters. Other predominant simple phenols and organic acids include ferulic acid, 4-hydroxycinnamic acid, 2-phenoxy ethanol, 3,4,5-trimethoxybenzoic acid, tyrosol, benzoic acid, phenyl acetic acid and D-3-phenyllactic acid. OFW are also characterised by the presence of vanillic acid, 2-phenoxyethanol, *trans*-cinnamic acid, *p*-hydroxybenzoic acid, 3,4-dimethoxybenzoic acid, cyclohexanecarboxylic acid, 3,4-dihydroxybenzoic acid, syringic acid and *p*-coumaric acid. The polyphenolic fraction is composed mainly of condensed tannins (Fig. 1.3), anthocyanins and humic acid-like compounds. The dark colour associated with olive wastewaters can be attributed to these larger polyphenolic compounds (Balice and Cera, 1984).

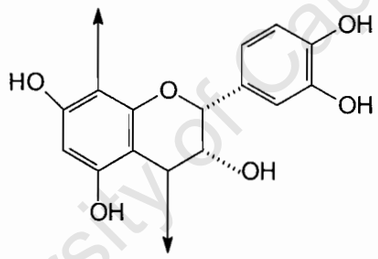



**Figure 1.3: The flavan-3-ol oligomeric unit, the fundamental structural unit of condensed tannins (proanthocyanidins) (Haslam, 1996).**

## 1.4 Treatment of olive wastewaters

### 1.4.1 Advanced oxidation processes (AOPs)

Although olive wastewaters comprise diluted juices of crushed and fermented olives, they are not easily biodegraded. Many of the constituents, such as the polyphenolic fraction, are decomposed at very slow rates (Rozzi and Malpei, 1996). For this reason many other non-biological treatment processes have been investigated.

Advanced oxidation processes (AOPs) are effective processes for treatment of phenolic containing wastewaters and there are a number of reports of significant organic removal from olive wastewaters (Table 1.1). These processes rely on the intermediacy of chemical initiators (i.e. free radicals) and energy (e.g. heat) to destroy the target pollutants (Mantzavinos and Kalogerakis, 2005). AOPs are based on the generation of very reactive and oxidising free radicals, in particular hydroxyl radicals that attack the organic compound and cause chemical decomposition (Benitez *et al.,* 2001). The production of the radicals is achieved by either the combinations of ozone with hydrogen peroxide ($H_2O_2$) and UV radiation, UV radiation with $H_2O_2$ (Glaze *et al.,* 1987) or with the combination of hydrogen peroxide with ferrous ions in the Fenton's reagent (Walling, 1975). Other processes involve photocatalysis, electrochemical oxidation, wet air oxidation and various combinations of these (Mantzavinos and Kalogerakis, 2005). AOPs can also be used as pre- or post-treatment in conjunction with biological treatments, both aerobic and anaerobic, in order to achieve maximum organic removal. The major drawback to these methods of treatment is that they are not as cost effective as biological methods, and because of this, they are not a viable option as a sole treatment process for the olive industry. Rivas *et al.* (2001) estimated that OMW treatment would cost USD 3.2/($m^3$ of wastewater treated and g/L COD removed). This is greater than that of conventional biological treatment of OMW by an order of magnitude. The major portion of the cost is due to $H_2O_2$ consumption and further studies would need to be conducted to optimise the dosage of Fenton's reagent used in order to minimise the costs (Mantzavinos and Kalogerakis, 2005).

Other non-biological methods of treatment include thermal processes such as distillation and evaporation. The main drawbacks to this type of treatment are related to post-treatment. In the case of OMW, the distillate is not pure and carries a significant amount of volatile compounds such as the volatile acids and alcohols. The disposal of the resulting concentrated paste is an additional problem. Combustion or incineration is another option but these give rise to atmospheric pollutants. In addition, these processes are expensive due to the high energy requirements.

**Table 1.1: Treatment of olive wastewaters with various AOPs.**

| Reference | AOP | Efficiency | Wastewater |
|---|---|---|---|
| Benitez *et al.*, 2001 | UV radiation | 35% COD, 20% total phenols removal after 6 h. Initial COD=2.51 g/L | OFW |
| | UV + $H_2O_2$ | 71% COD, 52% total phenols removal after 6 h. Initial COD=2.36 g/L | OFW |
| | Fenton's reagent | 65% COD removal after 6 h. Initial COD=2.22 g/L | OFW |
| | Photo-Fenton | 76% COD removal after 6 h. Initial COD=2.26 g/L | OMW |
| Rivas *et al.*,2001 | Fenton's reagent | 85 – 90% COD removal, 3-4 h. Initial COD=14.7 g/L | OMW |
| Vlyssides *et al.*, 2003 | Fenton/coagulation | 65% COD removal and 100% phenols removal after 2 h. Initial COD=80.3 g/L | OMW |
| Benitez *et al.*, 1999 | Ozonation | 22% COD removal after 8 h, initial COD=34 g/L | OMW |
| Israilides *et al.*, 1997 | Electrolysis over TI/Pt anode with NaCl as electrolyte | 93% COD, 80% TOC, 99.5% total phenols removal. Initial COD=178 g/L | OMW |
| Rivas *et al.*, 2001 | Supercritical wet air oxidation | 79% COD and 98% total phenols removal after 18s at 500°C, 25MPa. Initial COD=3.3 g/L | OMW |
| Chakchouk *et al.*, 1994 | Subcritical wet air oxidation coupled with Fenton | 77% COD and complete colour removal after 1 h at 200°C. Initial COD=50 g/L | OMW |
| Gernjak *et al.*, 2003 | Photo-Fenton with solar irradiation | 74% COD and 87% total phenols removal after 19 h. With flocculation as pretreatment, COD and phenols removal increased to 89 and 100% respectively. Initial COD=81 g/L | OMW |

### 1.4.2 Anaerobic treatment

A variety of anaerobic processes such as anaerobic contact, upflow anaerobic sludge blanket (UASB) and anaerobic filters have been applied to treat OMW (Hamdi, 1996) but there is limited literature on similar treatment processes for OFW. Advantages of anaerobic treatments of high strength wastewater include low costs for energy and chemicals and low sludge production. The seasonal production of OMW is not a disadvantage for anaerobic treatment because the decay rates of methanogens are very low and a digester can easily be restarted, even after months of shut-down (Lettinga *et al.,* 1980).

The major limitation to this process is the inhibition of the methanogenic bacteria caused mainly by the simple phenolic compounds present in the waste (Fig. 1.4) (Hamdi, 1992; Boari *et al.,* 1984). In addition to this, anaerobic degradation of the aromatic and long chain fatty acids in OMW presents a thermodynamic barrier and these compounds can only be removed by acetogenic bacteria associated with hydrogen oxidizing bacteria (Hamdi, 1992). Since the methanogens are severely inhibited by the phenolic compounds, this then represents a rate limiting step in the process. The inhibition of acetogenic bacteria results in an accumulation of hydrogen and acetate (Fig. 1.4) (Hamdi, 1992). High partial pressures of hydrogen inhibit the oxidation of the volatile fatty acids, long chain fatty acids and the aromatic fraction and therefore lead to the accumulation of ethanol, propionate and butyrate. This, in turn, leads to reduced acetate production which eventually results in the termination of the process (Dolfing and Tiedje, 1988).

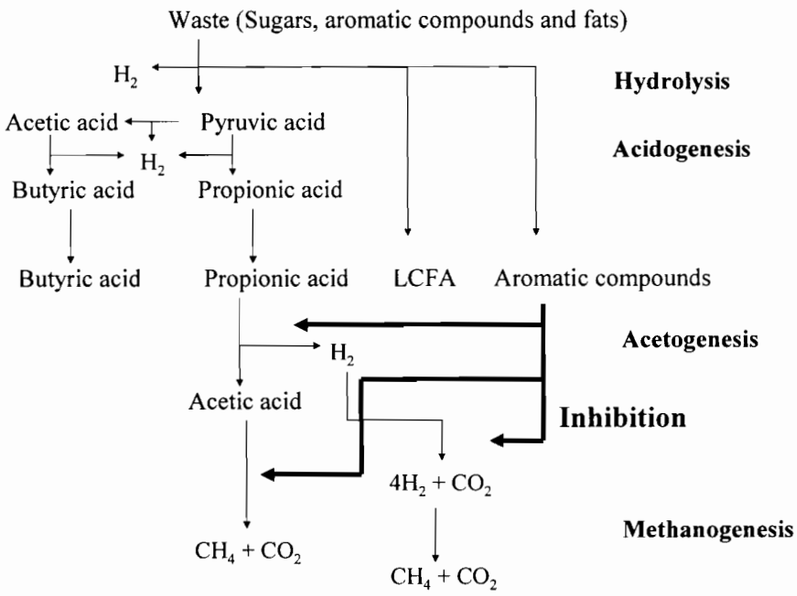



**Figure 1.4: Schematic representation of the anaerobic digestion of OMW and the inhibition by aromatic compounds (Hamdi, 1992). LCFA = long chain fatty acids.**

### 1.4.3 Aerobic treatment

Due to the toxic inhibition of methanogens by phenolic compounds, a pretreatment of OMW, using aerobic microorganisms to remove much of the simple phenolic fraction, has generated much interest and has been shown to improve the performance of the anaerobic purification process (Hamdi and Garcia, 1991; Borja *et al.,* 1992; Sayadi and Ellouz; 1992, Borja and Gonzalez, 1994; Borja *et al.,* 1995). As shown in Table 1.2, the organisms used to investigate the treatment of these olive wastewaters include bacteria, yeast and fungi, but predominantly fungi and yeast. It has been stated that OMW needs to be diluted 70 times in order to treat it by activated sludge (Balice *et al.,* 1988). It has also been shown that microrganisms, if acclimatised to the waste, are able to tolerate this harsh environment and can effectively biodegrade olive wastes. Activated sludge, from municipal treatment works, was investigated for its ability to biodegrade OFW, and it was found that the consortium required six days of stirring and agitation in the digester

containing black olive wastewater but was then able to remove up to 80% of the COD and 50% of the total phenols (Beltran-Heredia *et al.,* 2000). Acclimatisation of the bacteria, yeast or fungi to such a complex medium is essential to induce the production of the necessary enzymes capable of degrading or mineralising the target compounds. A variety of organisms would produce different enzymes and upregulate a range of metabolic pathways to deal with the mineralisation of numerous compounds, especially the phenolic compounds.

### 1.4.4 Combinations of treatment methods

Combinations of treatments have also been investigated. For instance, either AOP or aerobic treatment prior to anaerobic treatment could remove most of the toxic phenolic compounds and reduce the inhibition effect on methanogens. An aerobic treatment before AOP could reduce the amounts of reagents required, making the process more economical.

In a study of methane production during anaerobic treatment of pretreated OMW, Borja *et al.* (1995) found an increase in the yield coefficient of methane production. The OMW was aerobically pretreated with *Geotrichum candidum*, *Azotobacter chroococcum* and *Aspergillus terrreus* and this significantly reduced the COD and total phenol content. Similarly, Beltran-Heredia *et al.* (2000) found that the combination of an aerobic degradation step, followed by ozonation of black olive wastewaters, produced better phenol and COD removal efficiency than either of these processes individually. An investigation into the use of a chemical treatment prior to a biological one was investigated by Benitez *et al.,* (2001) on black olive fermentation wastewaters. The results reported concur with those of Borja *et al.* (1995) and they reported that the pretreatment increased the methane yield coefficient of the subsequent anaerobic process.

**Table 1.2: Degradation results obtained from the aerobic treatment of olive wastewaters.**

$X_{TP}$ = % removal of total phenols
$X_{COD}$ = % removal of COD

| Reference | Organism | $X_{TP}$ | $X_{COD}$ | Wastewater |
|---|---|---|---|---|
| Bertin *et al.*, 2001 | *Ralstonia* sp. and *Pseudomonas putida* | | | Synthetic mixture |
| Beltran-Heredia *et al.*, 2000 | Activated sludge (municipal) | 50 | 80 | OFW |
| Benitez *et al.*, 1997 | Activated sludge (municipal) | 90+ | 84 | Diluted OMW |
| Vinciguerra *et al.*, 1995 | *Lentinula edodes* | 66 | | Diluted OMW |
| D'Annibale *et al.*, 2004 | *L. edodes* | 88 | | OMW |
| | *Panus tigrinus* | | 79 | OMW |
| Aggelis *et al.*, 2003 | *Pleurotus ostreatus* | | | Diluted OMW |
| Fountoulakis *et al.*, 2002 | *P. ostreatus* | 78 | | OMW |
| Robles *et al.*, 2000 | *Penicillium* | 68 | 75 | OMW |
| Borja *et al.*, 1995 | *Geotrichum candidum* | 66 | 63 | OMW |
| | *Azotobacter chroococcum* | 90 | 75 | |
| | *Aspergillus terreus* | 94 | 74 | |
| Hamdi *et al.*, 1991 | *Aspergillus niger* | 58 | 61 | OMW |
| Assas *et al.*, 2000 | *Geotrichum candidum* | | 61 | OMW |
| Assas *et al.*, 2002 | *G. candidum* | | 65 | OMW |
| Garrido-Hoyos *et al.*,2002 | *A. terreus* | | 66 | OMW |
| Garcia *et al.*, 2000 | *Phanerochaete chrysosporium* | 92 | 75 | OMW |
| | *A. niger* | 76 | 73 | |
| | *A. terreus* | 64 | 63 | |
| Fadil *et al.*, 2003 | *G. candidum* | 0 | 45 | |
| | *Geotrichum* sp. | 47 | 55 | Diluted OMW |
| | *Aspergillus* sp. | 44 | 53 | |
| Martinez Nieto *et al.*, 1993 | *Candidia tropicalis* | 52 | 63 | |
| | *A. terreus* | | | OMW |
| | *Bacillus pumilus* | | | |

## 1.5 Bacterial degradation of industrial pollutants: their role in bioremediation

Bacteria play a vital role in the bioremediation of contaminated sites as they have the ability to transform potentially toxic compounds into non-toxic compounds biocatalytically, through the use of enzymes. Bacteria exist as mixed populations and are seldom found as pure cultures in the environment. It is often desirable to isolate and identify the naturally occurring microorganisms present in contaminated sites, such as the evaporation ponds, that have pollutant-degrading ability. These organisms can then be used in bioaugmentation or other forms of biological treatment since they show the ability to grow on carbon sources alternative to glucose, such as phenols.

Phenol and its derivatives are one of the main sources of environmental polluting industrial effluents from processes such as oil refineries, petrochemical plants, coal conversion plants, phenolic resin industries and agro-industry (Hinteregger *et al.,* 1992). Phenol is one of the most abundant aromatic contaminants in surface waters and contaminated soils and contributes to off-flavours in drinking and food-processing waters (Yang and Humphrey, 1975). Due to their toxicity to microorganisms, phenolic compounds can result in the termination of wastewater treatment processess by inhibition of microbial growth (Li and Humphrey, 1989). The metabolism of phenolics has been extensively investigated for a variety of microorganisms and studies of the major pathways for the catabolism of phenolics in bacteria reveal that, although the initial conversion steps are carried out by various enzymes, the compounds are then transformed into a limited number of central intermediates, such as protocatechuic acid and catechols (Tsirogianni *et al.,* 2004).

Hydroxylation of aromatics is the initial step in their degradation by bacteria (Dagley *et al.*, 1960). The enzyme phenol hydroxylase catalyses this initial reaction during phenol degradation by the insertion of oxygen at a position *ortho* to the existing hydroxyl group yielding catechol (Tsirogianni *et al.,* 2004). Ring cleavage of the resulting catechol can occur either between the two hydroxyl groups, initiating the *ortho*-pathway that leads to

succinyl-CoA and acetyl-CoA, or at the adjacent bond, initiating the *meta*-pathway that leads to pyruvate and acetaldehyde (Mason and Cammack, 1992). The enzyme that catalyses the dissimilation of the catechol intermediate is one of two dioxygenases, either catechol 1,2 dioxygenase or catechol 2,3 dioxygenase. These ring cleavage enzymes have been shown to be highly conserved between different bacterial species (Mishra *et al.*, 2001). Buswell (1975) reported that in *Bacillus stearothermophilus*, ring fission of the corresponding *o*-diol compounds produced by hydroxylation of phenol proceeded by the *meta*-cleavage route, catalysed by catechol 2,3-dioxygenase (Fig. 1.5). Olive wastewaters have proved to be difficult to remediate due to the presence of phenolic compounds known to be toxic at high concentrations. Moreno *et al.* (1987) conducted a study with OMW, in which soil samples were incubated with diluted (10% v/v) OMW in order to select for soil bacteria less susceptible to the antibacterial effect of the waste and, therefore, desirable for microbial processes designed to enable the re-use of the wastewater. They reported that it would not be difficult to develop, through adaptation, phenolic resistant strains from the isolated microbial groups, namely Coryneforms, *Enterobacteria*, *Acinetobacter* and *Pseudomonas,* but with the exception of Bacilli.

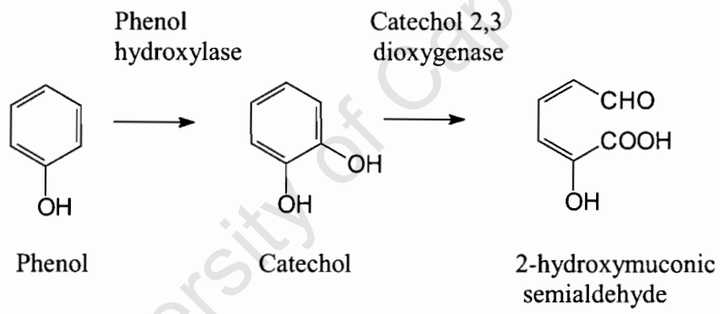



**Figure 1.5: Proposed reaction sequence for the metabolism of phenol by *B. stearothermophilus* (Buswell, 1975).**

## 1.6 Fungi and the ligninolytic enzyme systems

Cellulose, hemicellulose and lignin are synthesised and deposited in the plant cell wall and provide mechanical strength and rigidity to the stems of higher plants (Tuor *et al.,* 1995). White-rot fungi are well known for their ability to degrade wood and the enzymatic systems of selected wood degrading white-rot fungi have been elucidated to a large extent (Tuor *et al.,* 1995). The biochemistry and physiology of white-rot wood decay is not easily correlated to environmental conditions because there are a variety of fungi and only slight specificity towards the host trees, but white-rot fungi predominantly degrade wood from deciduous trees (Tuor *et al.,* 1995). Lignin is a three-dimensional, optically inactive phenyl-propanoid polymer randomly synthesised from coniferyl, *p*-coumaryl and sinapyl alcohol precursors (Sarkanen and Ludwig, 1971). Its biodegradation is mainly oxidative and is understood to be a multi-enzymatic process involving the mediation of small molecules and radicals (Leonowicz *et al.,* 1999). Fungi produce enzymes that initiate oxidative reactions within the lignin compound. This activates lignin to overcome an energy barrier and initiates a thermodynamically favoured oxidative fragmentation with no further control by the enzyme. The most important enzymes involved in lignin degradation are the phenoloxidases, lignin peroxidase (LiP; EC 1.11.1.14), manganese peroxidase (MnP; EC 1.11.1.13) and laccase (EC 1.10.3.2) (Leonowicz *et al.,* 1999). These enzymes have been identified in a number of different fungal strains (Table 1.3). These enzymes function cooperatively or separately (Leonowicz, *et al.,* 1999).

**Table 1.3: Some white-rot fungi and their oxidative enzymes (adapted from Tuor *et al.*, 1999).**

LiP = Lignin peroxidase
MnP = Manganese peroxidase
Lac = Laccase

| Microorganism | Enzymes | | |
|---|---|---|---|
| | LiP | MnP | Lac |
| *Trametes versicolor* | x | x | x |
| *Phanerochaete chrysosporium* | x | x | x (little) |
| *Pleurotus ostreatus* | x | x | x |
| *Lentinula edodes* | none | x | x |
| *Panus tigrinus* | none | x | x |
| *Pycnoporus coccineus* | none | none | x |

LiP and MnP oxidise their substrates by two consecutive one-electron oxidation steps which yield intermediate cation radicals. Laccase is a true phenoloxidase with broad substrate specificity (Tuor *et al.,* 1995). It catalyses the oxidation of phenols and phenolic lignin substructures by one-electron abstraction and forms radicals that can either repolymerise or lead to depolymerisation (Higuchi, 1989). Once the size of these extracellular enzymes had been established it became apparent that they were too large to penetrate wood (Evans *et al.,* 1994). Therefore in the case of fungal cells possessing high enough redox potential, low molecular weight mediators, were suggested to migrate from the enzymes to oxidise lignin or wood, thus acting as the enzyme "messengers" (Leonowicz *et al.,* 1999). Lignin degradation by fungi that produce mainly LiP utilise different pathways, and involve different mediators, from those that produce mainly MnP and laccase (Tuor *et al.,* 1995). Leonowicz *et al.* (1999) proposed a feedback-type enzymatic system for the transformation of lignocellulose involving LiP, MnP, laccase and various other enzymes, and suggested that the existence of other, still-to-be discovered enzymatic feed-back mechanisms was a possibility. They proposed that laccase oxidises lignin and produces quinoids and phenoxy radicals. Glucose oxidase (GO) is able to use these radicals as a source of oxygen with subsequent production of $H_2O_2$. This also serves as a means to protect the laccase enzyme, as excess quinones inhibit laccase. The production of $H_2O_2$ enables the functioning of LiP and MnP for further lignin decomposition into lower molecular weight fragments. The reduced

quinones, from the action of GO, produce the respective phenols that serve as the substrates for intracellular, ring-cleaving dioxygenases. The products obtained easily find their way to the Krebs cycle.

### 1.6.1 Bioremediation using white-rot fungi

The system of employing mediators allows for broad substrate specificity and hence, there is potential for other biotechnological applications for white-rot fungi. The non-specific nature of the ligninolytic enzymes makes them an attractive option for application in bioremediation because of their ability to degrade a wide range of pollutants. Pickard *et al.* (1999) performed a study on the metabolism of polycyclic aromatic hydrocarbons (PAHs) with white-rot fungi that had previously been shown to metabolise polychlorinated biphenyls (PCBs). Ten white-rot fungi grown in the presence of anthracene, pyrene and phenanthrene could metabolise these PAHs at a concentration of 5µg/ml on a growth medium known to support the production of MnP and laccase. The importance of MnP and laccase production was illustrated in a study on the degradation of anthracene and pyrene by the mycelia of *Phanerochaete chrysosporium, Trametes versicolor* and *Pleurotus ostreatus* (Novotny *et al.*, 2004). They report that the significantly lower levels of MnP, LiP and laccase produced by *P. ostreatus* compared to the rest correlated with its inability to remove PCBs from a liquid medium. Novotny *et al.* (2004) also studied the degradation of a number of synthetic dyes with the same ligninolytic fungi in liquid and soil. They reported that high levels of MnP correlated to good decolourisation of Reactive Orange 16 azo dye but not of Remazol Brilliant Blue anthraquinone dye. Robinson *et al.* (2001) reported degradation of an artificial textile dye effluent containing Cibacron Yellow C-2R, Cibacron Red C-2G, Cibacron Blue C-R, Remazol Black B and Remazol Red RB in equal amounts. The white-rot fungi *Bjerkandera adusta* and *Phlebia tremellosa* degraded up to 86 and 79% of the dyes respectively, and the enzymes LiP and laccase were produced at significantly higher levels.

Ligninolytic fungi have been intensively studied for application in the paper and pulp industry. Kraft pulping is conventionally used for the production of good quality

cellulose pulp subsequently used to make paper. Delignification is achieved by solubilisation in alkaline solutions. The residual lignin in the pulp is removed by treatment with chlorine. The effluent from this process is rich in chlorinated organic compounds with potential to pollute the environment (Taspinar and Kolankaya, 1998). It is therefore important that these industries reduce the chlorine usage in the kraft pulping process. *Trametes versicolor* was shown to brighten pulp and this was accompanied by a decrease in the kappa number (an indication of the lignin content) (Addleman *et al.*, 1995). Taspinar and Kolankaya (1998) conducted studies on enzymatic chlorine removal from a chlorine-based bleached kraft pulp using laccase from *T. versicolor* and concluded that the process was promising and could be considered as a novel biotechnological method for the dechlorination of any wastes containing organically bound chlorine compounds. Laccase has also been extensively used in the decolourisation of textile dye effluent from the textile industry. Commercial dyes are not uniformly susceptible to microbial attack in conventional aerobic treatment because of their unique and stable chemical structures (Wong and Yu, 1999). In a study of the enzymatic decolourisation of anthraquinone, azo and indigo dyes, Wong and Yu (1999) found that laccase from *T. versicolor* was able to directly oxidise anthraquinone while the decolourisation of azo and indigo dyes required the involvement of small metabolites as mediators, that are usually produced by the fungus. Therefore, this illustrates that laccase can oxidise compounds that are not substrates of laccase, with the use of mediators. The role of this mediation between laccase and non-substrate dyes can, however, be performed by substrate dyes, resulting in a potential enzymatic treatment process that does not require fungal cultures. Moldes *et al.* (2004) investigated the degradation of Phenol Red dye by laccase in the presence and absence of a mediator (HOBT) and they reported that the decolourisation was limited when the mediator was absent but the dye was completely degraded in the presence of mediator. The reason for this is that the radical produced from the oxidation of the mediator by laccase is able to oxidise a great variety of aromatic compounds and also reduce the polymerisation phenomenon due to its high redox potential.

The basidiomycetes *Trametes versicolor* and *Trametes pubescens* have been shown to be outstanding laccase producers and subsequently were able to efficiently bioremediate an

industrial wastewater containing, predominantly, phenols and cresols (Ryan *et al.,* 2005). This work illustrated the potential for *T. pubescens* in bioremediation since it has a superior laccase-producing ability.

### 1.6.2 Olive wastewater remediation using white-rot fungi

Many of the compounds present in olive wastewaters share structural similarity to that of lignin, the primary substrate for the ligninolytic enzymes. For this reason, white-rot fungi have been investigated for their ability to detoxify and decolourise OMW and OFW. There have been reports of the decolourisation of kraft mill effluents with various white-rot species (Sundman *et al.,* 1981; Bergbauer *et al.,* 1991) and therefore these organisms have been investigated to determine their effects on olive effluents. Table 1.2 indicates some of the organisms that have been investigated for their abilities to biodegrade the olive wastewaters and the majority of these are white-rot fungi. The involvement of the ligninolytic enzymes in the degradation of the constituents of olive wastewaters is controversial and researchers attribute the degradation of organic constituents and decolourisation to different enzymes.

The roles of LiP and MnP from *P. chrysosporium* in the decolourisation of OMW were investigated by Sayadi and Ellouz (1995) who reported that LiP was the limiting factor for the decolourisation of OMW. Vinciguerra *et al.* (1995) investigated bioconversion of OMW with *L. edodes* and results indicated enhanced MnP and laccase production which were responsible for the reduction of organic load. However, information shown in Table 1.3 indicates that this organism does not produce LiP. Higher levels of organic removal were obtained by D'Annibale *et al.* (2004) with the same organism on undiluted effluent but the only lignin-modifying enzyme detected was laccase. This is an interesting result as Vinciguerra *et al.* (1995) reported the necessity to dilute the waste to allow fungal growth, as have other researchers (Martirani *et al.,* 1996). In a study conducted by D'Annibale *et al.* (2004) with *P. tigrinus*, known to be an active laccase and MnP producer, it was found that the production of these enzymes was significantly affected by the initial organic load; a higher initial organic load lead to a delay in the removal of colour, organics and phenols, which correlated to the delayed production of the enzymes.

The fungus *P. ostreatus* was investigated for its ability to remove phenols from OMW and the enzyme studies revealed that only laccase was produced in the OMW cultures (Aggelis *et al.,* 2003). Toxicity studies conducted on the treated waste showed that decreased toxicity was not proportional to the removal of phenols, and it seems that some of the oxidation products from the laccase reaction (quinonoids, phenoxy radicals) may, in fact, be more toxic than the original phenolics.

Perez *et al.* (1998) reported that MnP, and its isozymes, were the predominant enzymes found in OMW decolourised by *Phanerochaete flavido-alba*. It was also reported that laccase was strongly induced in these solutions. This study is one of the first to suggest that laccase may play a key role, more so than LiP and MnP, in the biotransformation of OMW and its decolourisation. Following on from these studies, *Pycnoporus coccineus* was reported to be able to decolourise and reduce the COD of OMW without an additional carbon source. The fungus secreted only laccase even in the presence of compounds that promote the production of peroxidases (Jaouani *et al.,* 2003). Dias *et al.* (2004) investigated the activity and elution profile of laccase from a basidiomycete (Euc-1) in fermentation using OMW. It was observed that increased concentration of the waste resulted in decreased decolourisation but also that the presence of sugars has a positive effect on the level of decolourisation. This has been suggested before by other researchers (Sayadi and Ellouz, 1995) and is known as co-metabolism. More importantly, their study revealed the induction of a second isoenzyme of laccase that was not present in the nutrient control medium as well as a significant correlation between laccase activity over time with decolourisation. These observed results reinforce the assumption made by Perez *et al.* (1998) that the role of laccase may be key to the biodegradation and decolourisation of olive wastewaters.



### 1.7 The enzymology of laccase

Laccase is a copper-containing polyphenol oxidase first discovered in the Japanese lacquer tree, *Rhus vernicifera* over 100 years ago (Yoshida, 1883). Laccases are

multicopper oxidoreductases (benzenediol:oxygen oxidoreductases, E.C. 1.10.3.2) capable of catalysing the oxidation of phenolic and non-phenolic compounds using molecular oxygen as an oxidant (Eriksson *et al.,* 1990). Laccases have been identified in all groups of organisms including bacteria, fungi and insects (Mayer and Staples, 2002). In basidiomycete fungi, extracellular laccases are constitutively produced in small amounts (Bollag and Leonowicz, 1984), but their production can be increased considerably by a variety of inducing substances, mainly aromatic or phenolic compounds related to lignin or its derivatives, such as ferulic acid, 2,5-xylidine or veratryl alcohol (Gianfreda *et al.,* 1999). These enzymes (from various sources) have been sequenced and the homology of sequences is low between certain laccases and other well-studied copper oxidases but there is significant structural similarity (Cullen, 1997). The regions involved in copper binding are highly conserved (Cullen, 1997).

Laccases contain four copper atoms grouped into three types according to their spectroscopic and electronic paramagnetic properties (EPR) (Fig. 1.6) (Messerschmidt, 1997). The blue colour (Abs ~ 600 nm) of the enzyme can be attributed to the type 1 copper (Cu1). This copper atom is situated at the T1 site and coordinates a cysteine (Mougin *et al.,* 2004). The T2 site contains a type 2 copper (Cu2), has no strong visible absorption spectrum but does have an EPR signal (Mougin *et al.,* 2004; Mester and Tien, 2000). Site T3 contains copper atoms Cu3a and Cu3b and has no EPR signal but shows an absorption maximum at 330 nm (Mester and Tien, 2000). In the T3 site, Cu3a and Cu3b are strongly coupled and connected by a hydroxide bridge (Mougin *et al.,* 2003; Mester and Tien, 2000). The copper atoms at sites T2 and T3 form a trinuclear cluster and it is at this site where dioxygen is reduced to form water (Mester and Tien, 2000). The mechanism of action of laccases can be described as follows: the mononuclear T1 site extracts electrons from a reducing substrate bound to the active site and mediates the transfer of these electrons to the T2/T3 site, where dioxygen is reduced to form water (Fig. 1.6) (Messerschmidt, 1997). The radical compounds, formed by the oxidation of the substrate, are released from the active site. These radicals are very reactive and able to interact with similar radicals from other laccase-catalysed reactions, or with other

compounds in the vicinity. These latter reactions induce numerous, non-enzyme catalysed reactions (Messerschmidt, 1997).

Laccases can catalyse the direct oxidative coupling of xenobiotics, as was illustrated in liquid media by Dubroca *et al.* (2003), who reported the efficient conversion of nonylphenol into polymeric compounds. Laccases can also catalyse the indirect oxidation of compounds that are non-phenolic but in this case they require the presence of a redox mediator, which can be natural or synthetic. The mediator promotes or facilitates enzyme action by increasing its oxidation potential (Mougin *et al.,* 2003).

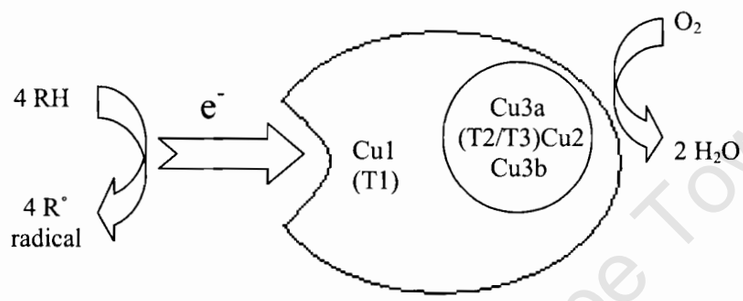



**Figure 1.6: Schematic representation for the mechanism of action of fungal laccases (Mougin *et al.*, 2003).**

In white-rot fungi, laccases are typically produced as multiple isoenzymes (Bollag and Leonowicz, 1984) encoded by gene families. It has been suggested that genes encoding various isoenzymes are differentially regulated (Mansur, 1998), with some being constitutively expressed and others being inducible (Bollag and Leonowicz, 1984; Soden and Dobson, 2001). Mansur *et al.* (1998) report three laccase genes, *lcc1, lcc2* and *lcc3* from Basidiomycete I-62 (CECT 20197), two of which are inducible under certain conditions and the third being uninducible and repressible. *T. pubescens* has only recently been identified as an excellent laccase producing fungus and laccase activity has been

reported to be as high as 740 U/ml (Galhaup *et al.*, 2002) in a defined medium, which is among the highest activities reported for laccase production to date.

### 1.7.1 Biotechnological application of laccase

Laccase is an enzyme with much appeal for industry. Its broad substrate specificity is the key characteristic that makes this enzyme so versatile. The two major industries that have found use for laccase are the textile and the paper-and-pulp industries. It has been established that laccase can bleach several textile dyes including triarylmethane, indigoid, azo and anthraquinone dyes (Wong and Yu, 1999; Abadulla *et al.,* 2000). The use of cell-free laccase as enzyme catalysts to delignify and bleach the pulp from the paper industry has been shown to work but requires the addition of mediators when the fungus is not present. This is because the fungi usually secrete a mixture of these mediators (Call and Mucke, 1997).

### 1.7.2 Olive wastewater remediation using laccase

Since the key enzymes thought to be involved in the biodegradation of the constituents of olive wastewaters have been identified, it seems logical that the application of these in a cell-free form would be a worthwhile investigation. This has been done by several researchers. Phenol oxidation results in highly reactive free-radicals that promote polymerisation of phenolic substrates which may be removed by filtration, flocculation or precipitation. Greco *et al*. (1999) investigated the effect of laccase-containing olive husk on the biodegradation of OMW and compared that to laccase from *T. versicolor*. They found that the free laccase was less effective in phenol removal than olive husk. Also, they identified the economical limitation of enzyme loss as a barrier to the practical application of free laccase and suggested enzyme immobilisation as a means to overcome this barrier. In a study conducted by D'Annibale *et al.* (2000), the immobilisation of free laccase was investigated. Laccase from *L. edodes* was immobilised on Eupergit® C and a significant reduction of total phenols, in particular *o*-diphenols, from OMW was observed.

The laccase from *L. edodes* was investigated for its ability to reduce the phytotoxicity of OMW (Casa *et al.,* 2003). In this study, total phenols were reduced from 3.7 – 1.3 g/L (65%) in 12 hours and the *ortho*-diphenols were reduced from 1.2 – 0.14 g/L (86%) using 0.8 U/ml OMW. These reductions were explained by the polymerisation of phenolic compounds as revealed by size exclusion chromatography. Durum wheat seeds were germinated in the presence of untreated or enzyme-treated OMW and an increase in germinability was observed. This indicated that the phytotoxicity of olive wastewaters could be significantly reduced by a biological treatment able to remove the phenolic components.

Laccase from *Pycnoporus coccineus* was investigated for its role in the degradation of different fractions of OMW. The low and high molecular mass fractions were separated by ultrafiltration, and analysed by gel filtration before and after enzymatic treatment. Polymerisation occurred due to the production of radicals from the oxidation of monomeric phenolic compounds. This phenomenon was reduced in the presence of a mediator which is thought to prevent repolymerisation. It was concluded that laccase played an important role in the degradative process (Jaouani *et al.,* 2005).

## 1.8 Beneficial utilisation of olive wastewaters

The large volumes of wastes generated make the identification of any possible benefits of these wastes very desirable. Olive wastes are a vast source of organic matter which could be recycled back into the environment as well as containing many other compounds with possible economical value or value as substrates for the production of other metabolites (Ramos-Cormenzana *et al.,* 1995). Literature regarding OMW is plentiful but, again, it is limited with regards to fermentation brines.

### 1.8.1 The use of olive wastewaters as a fertilizer

Land application is the oldest method for the disposal of wastes and serves two major purposes, firstly, waste disposal and secondly the recycling of waste components (Cabrera *et al.,* 1996). The high organic load and substantial amounts of plant nutrients,

and the fact that it is a low cost source of water makes OMW attractive for use as a fertilizer (Cegarra *et al.,* 1996). Chatjipavlidis *et al.* (1996) found that OMW constitute a rich and highly selective nutrient medium favouring the proliferation of nitrogen fixing bacteria of the genus *Azotobacter*. In an investigation by Balis *et al.* (1996), soil that had been enriched with OMW had acquired stability in structure, resistant to soil-borne root pathogenic fungi and improved fertility value. This was illustrated by growth trials in pots with lettuce, wheat, tomato, sunflower and maize (Flouri *et al.,* 1990; Balis *et al.,* 1991). Still, in concentrated form, OMW is a source of pollution. Cabrera *et al.* (1996) reported that leaching of mobile species such as $Na^+$ and $NO_3^-$ is likely to occur below the 1m layer of calcareous soils treated with OMW possibly leading to pollution of the water table and thus careful attention must be paid to the hydrogeological conditions of the soil. They concluded that land treatment in soils with low permeability might be a feasible option for OMW.

The fermentation waters are burdened with another obstacle and that is the high salt content. The addition of this waste to soil is by no means a suitable approach to its disposal as the salt would cause significant concentration gradients and the soil microflora would experience osmotic stress. The salt from the wastewaters after evaporation could reach concentrations that are toxic to plants. This phenomenon, known as soil salinisation, could leave the land sterile.

### 1.8.2 Olive wastes as a source of biologically active compounds

As outlined earlier, olive wastewaters comprise an abundance of different organic compounds from simple phenolics and phenolic acids to complex polyphenols and these have also been noted to have significant antimicrobial action. Puupponen-Pimia *et al.* (2005) report that compounds present in various berries, and which are also present in olive wastewaters, may have important applications in the food industry as natural antimicrobial agents. In a study conducted by Aziz *et al.* (1998) on the comparative antimicrobial and antifungal effects of *p*-hydroxybenzoic acid, vanillic, caffeic, protocatechuic, syringic and *p*-coumaric acids, oleuropein and quercetin (found in olive wastewaters), they have reported that these compounds showed wide antimicrobial

activity to *Escherichia coli, Klebsiella pneumoniae, Bacillus cereus, Aspergillus flavus* and *Aspergillus parasiticus* and may selectively inhibit pathogenic bacteria and aflatoxigenic moulds. They concluded that their application as antimicrobial agents is a practical possibility.

One of the more plentiful compounds present in the wastewaters, hydroxytyrosol, has for many years been studied because of its remarkable antioxidant and biological properties, such as antibiosis and plant growth regulation (Capasso *et al.,* 1994). Capasso *et al.* (1994) set about purifying the compound from the waste. Hydroxytyrosol was purified by means of a chromatographic method of filtration and lyophilisation, chromatography on silica gel and further chromatography on silica gel plates. Chemical transformation of the product formed a more stable triacetyl derivative, resistant to oxidation by air and light. Its preparation from the natural source for laboratory use is economical and convenient (Capasso *et al.,* 1994). Manna *et al.* (1999) investigated the protective effect of hydroxtyrosol on hydrogen peroxide ($H_2O_2$)-induced oxidative alterations in human erythrocytes. Human erythrocytes are exposed to oxidative hazard because of their function as oxygen carriers. They reported that hydroxytyrosol prevented oxidative alterations, providing protection against peroxide-induced cytotoxicity in erythrocytes. The mechanism of action of hydroxytyrosol is not known but O'Dowd *et al.* (2004) reported that hydroxytyrosol could exert its antioxidant effect by scavenging $H_2O_2$ but not superoxide anions released during the respiratory burst.

Previous studies have shown that UVA radiation induces severe skin damage through the generation of reactive oxygen species. Hydroxytyrosol was reported to have a protective effect in UVA-irradiated human melanoma cells, which was dose dependent (D'Angelo *et al.,* 2005).



## 1.9 Project objectives

A study was undertaken in order to investigate the bioremediation of black olive fermentation wastewaters. The first objective of this study was to screen strains isolated from evaporation ponds for their ability to reduce the phenolic fraction of black olive

fermentation wastewaters from local plants, and thereby reduce the toxicity of the wastes. The rationale behind the use of a strain isolated from an olive waste-polluted site is that the organism would already be acclimatised to the harsh environment. The organism would be in the correct metabolic state and the production of enzymes for the degradation of phenolic compounds would be induced. This project was a collaborative project with the ARCAM Research Group at the University of the Western Cape (UWC). The objective of our collaborators was to genetically modify the isolated strain in order to increase the strain's ability for the reduction of the organic load of this olive wastewater.

The second objective was to compare the abilities of the locally isolated strain and that of a commercial ligninolytic, white-rot fungus, *Trametes pubescens*, for bioremediation of black olive fermentation wastewater (olive wastewater). In addition to this, the fungal strain was to be further investigated to determine the effect of increased concentration of olive wastewater, and to correlate the growth of the fungus in this waste medium with laccase production. This is important because laccase is thought to play a role in the biodegradation of olive wastewaters.

Finally, laccase, isolated from *T. pubescens*, was investigated for its ability to detoxify the wastewater in the absence of the fungus. Laccase isolated from various white-rot fungi has been applied in bioremediation investigations of OMW with relative success, and therefore it was important to investigate its effect on black olive fermentation wastewaters. If the free enzyme is able to reduce the toxic phenolic fraction it may prove to be a practical pretreatment method to an anaerobic treatment with a significantly shorter treatment time than a whole cell aerobic treatment process.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Characterisation of olive wastewater

### 2.1.1 Olive wastewater used

The olive wastewater used in this study was obtained from the Buffet Olives plant in the Western Cape. The wastewater was taken directly from the black olive fermentation tanks before disposal into the evaporation ponds. Large quantities (25 L) of wastewater were collected periodically. Aliquots of the wastewater were kept at 4°C for short term use and the rest at -20°C for long term storage. Any mention made to "olive wastewater" throughout this chapter and the following chapters refers to this brined black olive wastewater, unless otherwise stated.

### 2.1.2 Chemical Oxygen Demand (COD) determination

Analyses were performed using the Merck COD reagent set (range 500 – 10 000 mg/L) in conjunction with a digestion block and a Nova Spectroquant 60 photometer. Firstly 2.2 ml of solution A and 1.8 ml of solution B were pipetted into an empty reaction cell and mixed. 1 ml of diluted (1:5) sample solution was added and mixed. The reaction solution was then heated for two hours at 148°C in the heating block. Samples were removed, cooled and measured. Potassium hydrogen phthalate (KHP, Merck) was used as a standard at concentrations of 500 and 1000 mg/L (COD). A blank was prepared with $dH_2O$ in place of sample solution.

### 2.1.3 Total phenols determination

Total phenols concentrations, in the olive wastewater, were measured colorimetrically using Folin-Ciocalteu reagent according to the procedure of Cheung *et al.* (2003). 400 µl reagent was added to 400 µl sample (diluted 1:20). After three minutes 400 µl $Na_2CO_3$ (20 % v/v) solution was added and the reaction mixture was made up to 4 ml with $dH_2O$. Samples were read at 765 nm in a Unicam Helios Alpha spectrophotometer. Gallic acid

standards were prepared (0 – 100 mg/L) and all total phenol concentrations are reported as gallic acid equivalents (GAE).

### 2.1.4 Liquid/liquid extraction of simple phenols from olive wastewater with ethyl acetate

Simple phenolics were extracted from raw olive wastewater samples using 3 x 1 v/v ethyl acetate. The separated organic phases were pooled, dried over anhydrous $Na_2SO_4$ and concentrated to dryness in a rotary evaporator. The residue was resuspended in 0.1 vol methanol. The phenolic extract was analysed by high performance liquid chromatography (HPLC) to determine individual phenolic components.

### 2.1.5 High performance liquid chromatography (HPLC) for the determination of individual phenolic compounds present in olive wastewater

Reverse phase HPLC was used to elucidate the range of individual phenolic compounds in olive wastewater, using a Merck Hitachi L-7000 series equipped with auto sampler/auto injector (20 µl). The mobile phase consisted of 5% formic acid (solvent A) and methanol (solvent B) and a gradient was installed to achieve the following ratios: 15% B at 3 min, 25% B at 13 min, 30% B at 25 min, 35% B at 35 min, 40% B at 39 min, 45% B at 42 min, 45% B at 45 min, 47% B at 50 min, 48% B at 60 min, 50% B at 64 min and 100% B at 66 min, at a flow rate of 1 ml/min. The column used was a Waters Spherisorb S5 ODS1 4.6 x 250 mm, with a Phenomenex guard column. UV detection was performed at 280 nm. Individual compounds were identified by comparison of retention times against hydroxytyrosol and tyrosol. Hydroxytyrosol was obtained by C. Garcin by the method of Garcia *et al.* (1996) and tyrosol was purchased from Merck.

### 2.1.6 pH determination

pH was measured using a calibrated Corning 240 pH meter. Determinations were performed by placing the probe directly into the sample.

### 2.1.7 Conductivity determination

The conductivity of the waste was measured using a Lutron CD-4301 conductivity meter.

### 2.1.8 Total nitrogen content

$NH_4$-N was determined by both ion chromatography and colorimetric assay according to Chaney and Marbach (1962). Total nitrogen was measured using a Hanna reagent set (HI 93767 low range) in conjunction with a Hanna C214 bench photometer.

### 2.1.9 Sugar content

Reducing sugars were determined using the dinitrosalicylic acid colorimetric method according to Miller (1959). Glucose was used as a standard at concentrations from 0 – 1 g/L.

### 2.1.10 Total, suspended and dissolved solids

Total solids were determined by evaporating a well mixed sample in an oven at 103 - 105°C, until constant weight was achieved. The samples were cooled to room temperature in a dessicator before weighing. Dissolved solids were determined in the same manner after a sample had been passed through a filter (Millipore 0.45µm nitrocellulose). Suspended solids are the portion retained by the filter. These were also measured gravimetrically, after drying, by subtracting the initial weight of the filter.

### 2.1.11 Lipid content

Lipids were determined by extraction with chloroform: methanol (2:1) using the Folch general procedure (Folch, 1957).

### 2.1.12 Total organic carbon

Total organic carbon (TOC) was measured using a SGE Anatoc Series II analyzer.

## 2.2 Isolation and screening of microorganisms capable of growth on olive wastewater

### 2.2.1 Isolation of wild type strains from olive wastewater

The isolation of wild type strains was performed in collaboration with A. van Schalkwyk (Department of Biotechnology, University of the Western Cape). Aliquots of olive

wastewater samples collected from various evaporation disposal ponds located at Buffet Olives were plated onto 25% (v/v) olive wastewater agar (5 g/L yeast extract (YE), 16 g/L agar) and incubated at 30 and 37°C for 24-96 hours. Isolates with unique colony morphology and colour were picked and repeatedly subcultured on 25% (v/v) olive wastewater agar, in order to obtain pure cultures. 35 isolates were obtained and these were maintained on 25% (v/v) olive wastewater agar (5 g/L YE, 16 g/L agar, pH 6) or 25% olive wastewater broth (5 g/L YE, pH 6) and stored at 4°C.

### 2.2.2 Growth and selection of isolates for further studies

100 µl of each of these cultures was spread on 25% (v/v) olive wastewater agar (5g/L Yeast extract and 16g/L agar) plates. The plates were incubated at 28°C for 24 to 96 hours and growth was assessed by monitoring colony formation. Those cultures that showed the best growth with respect to colony numbers were selected for degradation studies.

## 2.3 Initial studies for the degradation of olive wastewater by selected isolates

### 2.3.1 Inoculum preparation

The selected organisms (AS-1, AS-3, AS-9, AS-12, AS-28 and AS-35) were maintained on 25% (v/v) olive wastewater agar plates (5 g/L YE). Nutrient broth with 2% (v/v) olive wastewater was prepared in Erlenmeyer flasks (250 ml, 100 ml working volume) and inoculated with a single loop from the agar plates. These were incubated at 28°C, with orbital shaking at 150 rpm for 24 hours.

### 2.3.2 Culture conditions

Culture experiments were carried out in 500 ml Erlenmeyer flasks with 250 ml 25% (v/v) olive wastewater with added nitrogen source (5 g/L YE) and inoculated with 10% (v/v) inoculum. All media were sterilized at 121°C for 20 min prior to inoculation. Flasks were incubated at 28°C with orbital shaking at 150 rpm. Samples (5 ml) were taken aseptically every 24 hours for analysis of total phenols and pH determinations as described previously. Experiments were performed in triplicate.

## 2.4 Olive wastewater degradation by strain AS-35 in a stirred tank reactor

### 2.4.1 Inoculum preparation

These were prepared as discussed in section 2.3.1.

### 2.4.2 Fermentation of AS-35 in 25% (v/v) olive wastewater in the BioFlo 110 fermenter

These experiments were carried out in a New Brunswick BioFlo 110 fermenter with a working volume of 5 L (Fig. 2.1). The fermenter has a removable pH probe that connects to the Primary Control Unit (PCU) and pH was monitored continuously throughout the fermentation. The probe was calibrated manually before the entire vessel was sterilized by autoclaving (20 min, 121°C), prior to inoculation. A 10% (v/v) inoculum was added to the 4.5 L of sterilised media (25% (v/v) olive wastewater, 5 g/L YE) through a port in the fermenter head plate and the temperature was set to 28°C. Agitation was set to 250 rpm and the air flow rate at 1 v/v/min (Dhouib *et al.,* 2003). Samples (20 ml) were collected aseptically every 24 hours by means of the sampler system (Fig. 2.2) for total phenols analysis.

### 2.4.3 Fermentation of AS-35 in 25% (v/v) olive wastewater in the BioFlo 110 at constant pH

The above experiment was repeated at controlled pHs of 6 and 7. The pH of the medium was adjusted to 6 and 7 with 1M NaOH before sterilising and kept constant with the addition of 0.5M HCl.

### 2.4.4 pH

This was measured automatically with the unit pH probe connected to the PCU.

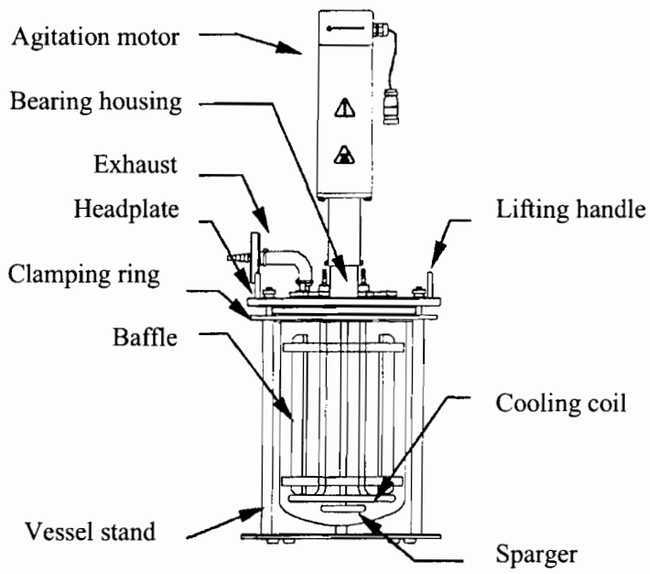



**Figure 2.1: Schematic representation of the BioFlo 110 fermenter (New Brunswick Scientific, BioFlo 110 Modular fermenter, Manual No. M1273-0054).**

## 2.5 Comparison of the olive wastewater degradation and decolourisation capabilities of strain *B. megaterium* AS-35 and *Trametes pubescens*

### 2.5.1 Identification of isolate AS-35 using molecular techniques

This organism was identified as *Bacillus megaterium* using 16S rRNA gene analysis and API 50 CHB biochemical tests by A. van Schalkwyk (UWC). *B. megaterium* AS-35 was routinely cultured in Luria-Bertani broth (5 g/L YE, 10 g/L tryptone, 10 g/L NaCl, pH 4).

### 2.5.2 Maintenance and inoculum preparation of *B. megaterium* AS-35

Cultures of *B. megaterium* AS-35 were received from A. van Schalkwyk and the culture medium was altered to a simpler nutrient/waste agar medium (1.5% (w/v) nutrient broth, 2% v/v olive wastewater, 16 g/L agar) on which the organism was maintained. The bacterial inoculum was prepared as described in section 2.3.1.

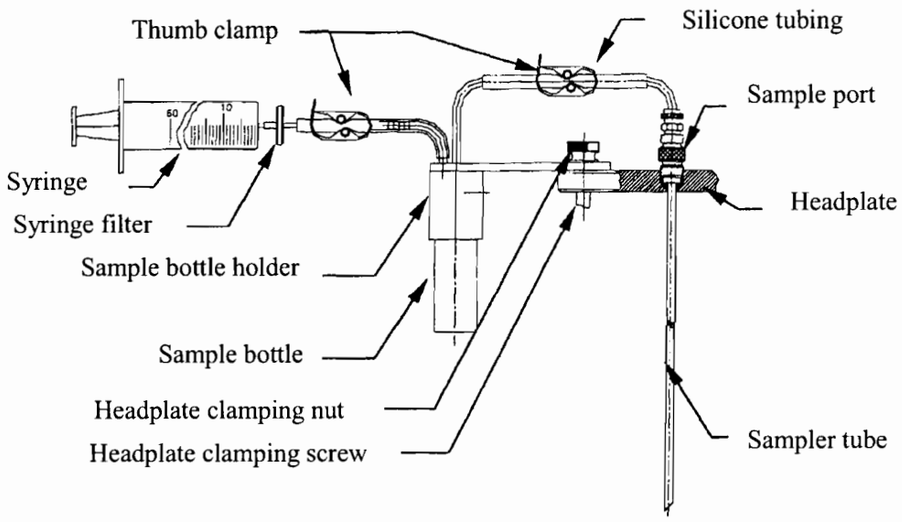



**Figure 2.2: Schematic representation of the sampler system of the BioFlo 110 fermenter (New Brunswick Scientific, BioFlo 110 Modular fermenter, Manual No. M1273-0054).**

### 2.5.3 Maintenance and inoculum preparation of *T. pubescens*

*T. pubescens* (CBS 696.94) was originally obtained from the Boku Institute in Austria and was maintained on 3% (w/v) malt extract agar at 4°C, subcultured every two months. A single agar plate was homogenised in 200 ml of sterilised $dH_2O$ with a benchtop homogeniser and 50 ml of this homogenate was used to inoculate 500 ml of Trametes Defined Media (TDM) with 2% (v/v) olive wastewater added as inducer. TDM was adapted from Addleman and Archibald (1993) (appendix A). The medium was initially developed for optimised growth and laccase production of *T. versicolor* (Ryan *et al.*, 2005) and is a carbon and nitrogen sufficient medium. The medium was simplified by the omission of thiamine. All media were sterilised at 121°C for 20 minutes prior to inoculation. These flasks were incubated at 28°C with rotary shaking at 175 rpm. This speed was found to produce small, dense pellets. Four-day-old pellets from these cultures were used to inoculate the experimental vessels.

### 2.5.4 Experimental design for the fermentation of *B. megaterium* AS-35 and *T. pubescens* for the biodegradation of olive wastewater in three different vessel configurations

Three different reactor configurations were investigated: shake flasks, a pneumatic reactor (airlift) with internal draught tube and a continuously stirred tank reactor (CSTR). Fermentations were conducted with *B. megaterium* AS-35 and *T. pubescens* in each of the three different configurations with a 10% (v/v) dilution of olive wastewater.

#### 2.5.4.1 Shake flask experiments to determine growth of the above organisms and total phenols removal from olive wastewater

Experiments were carried out in 500 ml Erlenmeyer flasks with 250 ml of 10% (v/v) diluted olive wastewater (5 g/L YE added) and inoculated with 10% (v/v) inoculum of *B. megaterium* AS-35 or *T. pubescens*. The flasks were incubated at 28°C with orbital shaking at 150 rpm for the duration of the experiment. Samples (5 ml) were taken aseptically for analysis.

#### 2.5.4.2 Batch bioreactor experiments for comparison of growth of the above organisms and total phenols removal from olive wastewater in various vessel configurations

***a) Stirred tank reactor***

The New Brunswick BioFlo 110 fermenter was used in this study. 4.5 L of 10% (v/v) olive wastewater (5 g/L YE added) was inoculated with 500 ml (10 % v/v) of the inoculum preparations of *B. megaterium* AS-35 or *T. pubescens*. The agitation was set to 250 rpm and air flow rate at 1 v/v/min. Samples (20 ml) were drawn aseptically by means of the sampler system for analysis. The pH was measured automatically for the duration of the experiment.

***b) Airlift reactor***

The reactor (Fig. 2.3) used was the same as that used in a previous study for the bioremediation of stripped gas liquor effluent (Ryan *et al.,* 2005). This reactor has a

height of 500 mm, aspect ratio (H/D) of 4.5, internal draught tube diameter of 55 mm and a working volume of 3.8 L. Experiments were conducted at 28°C with a constant air flow rate of 1.5 L/min. The reactor was filled with 3.45 L of 10% (v/v) olive wastewater (5 g/L YE added) and inoculated with 350 ml (10% v/v) of inoculum. Samples (10 ml) were drawn aseptically for analysis.

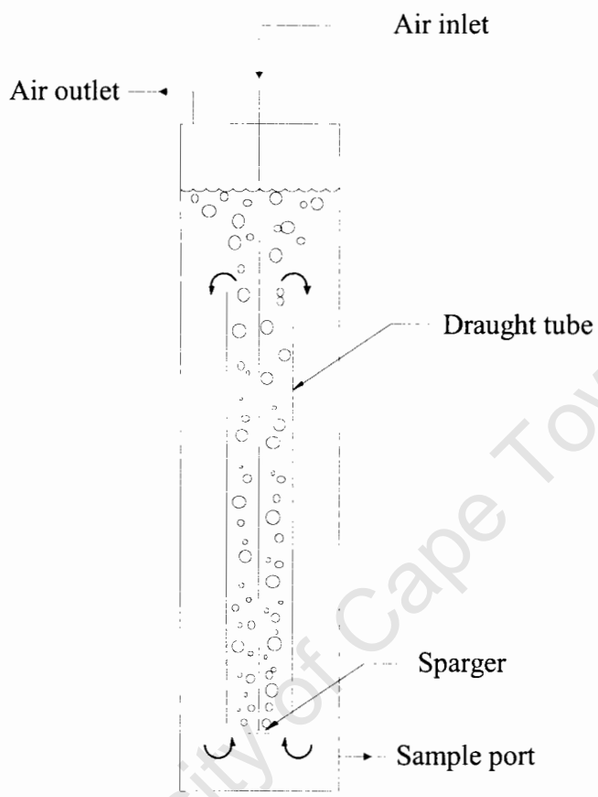



**Figure 2.3: Schematic representation of the airlift reactor, with internal draught tube, used in this study.**



### 2.5.5 Biomass determination

Samples from each of the experiments were filtered through pre-weighed Millipore nitrocellulose filters (0.45 μm) using a vacuum pump and the filtrate was collected and

stored for later analysis. Filters were then placed in an oven at 80°C for 24 hours for drying, and cooled in the dessicator for 1 hour before weighing to determine the dry mass. From these dry mass determinations, the maximum specific growth rates were determined as follows:

$$r_x = \frac{dX}{dt} \qquad (1)$$

$$\mu_{max} = \frac{r_x}{X} \qquad (2)$$

$r_x$ = cell growth rate (mg/L. $h^{-1}$)

$dX/dt$ = cell accumulation

$\mu_{max}$ = maximum specific growth rate ($h^{-1}$)

### 2.5.6 Molecular weight distribution analysis of phenolic compounds from olive wastewater

The molecular weight distributions of phenolic compounds from the samples taken from the experiments above were monitored using size exclusion chromatography with an AKTA Prime fraction collector. The stationary phase was Sephadex G-50 (medium) in a 70 x 26 mm column (Amersham) and the mobile phase was $dH_2O$ at a flow rate of 1 ml/min. UV detection was performed at 280 nm. The sample (2 ml) was injected and automatically loaded onto the column and 10 ml fractions were collected during elution. The retention time calibration standards used were Dextran blue (Fluka, 1 g/L, Mr = 2000 kDa) and gallic acid (Sigma, Mw = 170 kDa). A solution of Humic acid (Fluka, 2 g/L) was also run through the column for comparison of the high molecular weight polyphenolics present in the wastewater.

### 2.5.7 HPLC analysis of samples for the determination of individual phenolic compounds in olive wastewater

Reverse phase HPLC was used to elucidate the range of individual phenolic compounds from the samples taken before and after fermentation of olive wastewater, using a Merck

Hitachi L-7000 series equipped with auto sampler/auto injector (20 µl). The smaller monophenols were separated by isocratic elution with a mobile phase of water/methanol/acetic acid (80:20:2.5) at a flow rate of 1 ml/min. The column used was a Waters Spherisorb S5 ODS1 4.6 x 250 mm, with a Phenomenex guard column. UV detection was performed at 280 nm.

### 2.5.8 Colour analysis of the olive wastewater to determine decolourisation

Samples from the experiments conducted were centrifuged at 16 060 g for 3 minutes and the colour of the wastewater was determined spectrophotometrically on the supernatants of diluted samples (1:10) at 465 nm with a Unicam Helios Alpha spectrophotometer (Robles *et al.*, 2000).

## 2.6 Further investigation of the biodegradation of olive wastewater by *T. pubescens*

### 2.6.1 Inoculum preparation

The inocula for each of the experiments were prepared as discussed in section 2.4.1.

### 2.6.2 Correlating growth, laccase production and total phenols reduction in 10 and 25% (v/v) olive wastewater with *T. pubescens*

This investigation was undertaken to determine the effect of increased concentration on the degradation of olive wastewater by *T. pubescens* and to correlate growth, laccase production and the removal of total phenols. Experiments were conducted in 1L shake flasks with 250 ml of 10 and 25% (v/v) olive wastewater medium (5 g/L YE added). All media were sterilised (20 min, 121°C) prior to inoculation. A 10% (v/v) inoculum was used to inoculate each of the flasks. Experiments were performed in triplicate.

### 2.6.3 Laccase activity determination

Laccase activity was assayed using the procedure of Roy-Arcand and Archibald (1991). The reaction mixture contained 2.5 ml 0.1 M sodium acetate buffer (pH 5), 0.33 ml 5 mM ABTS (2,2'-azinobis 3-ethylbenzthiazoline-6-sulfonic acid) and 0.17 ml enzyme sample. Oxidation of ABTS was measured by determining the increase in absorbance of the

mixture at 420 nm ($\varepsilon$ = 36 000 $M^{-1}$) with a Unicam Helios Alpha spectrophotometer. One unit (U) of enzyme activity was defined as the amount of enzyme required to oxidise 1 µmol of ABTS per min.

## 2.7 Use of laccase isolated from *T. pubescens* for bioremediation of olive wastewater

### 2.7.1 Production of laccase in TDM by *T. pubescens* using olive wastewater (2% v/v) to induce production

Laccase was produced in 1 L Erlenmeyer flasks with 500 ml of TDM, 2% (v/v) olive wastewater, inoculated as discussed in section 2.3.1. Flasks were incubated at 28°C with orbital shaking at 150 rpm, in the dark, for 96 hours. The biomass was removed by centrifugation at 10 000 rpm for 5 minutes and the supernatant, containing extracellular enzymes, was freeze-dried, yielding the crude extract, which was stored at 4°C in powder form.

### 2.7.2 Laccase activity determination

This was determined as described in section 2.6.3.

### 2.7.3 Protein determination

Protein concentrations were determined using Bradford reagent from Sigma Chemical Co. The reaction mixture contained 0.05 ml of sample or standard (BSA standards, 0 – 1 mg/ml) and 1.5 ml of reagent. The reaction mixtures were mixed and left to incubate at room temperature for 20 minutes before absorbances were read at 595 nm with a Unicam Helios Alpha spectrophotometer.

### 2.7.4 Characterisation of laccase from *T. pubescens* by SDS-PAGE

The freeze-dried crude extract of laccase was resuspended in 0.1 M sodium acetate buffer (pH 5), dialysed against Na-acetate buffer (0.1 M, pH 5) overnight to concentrate the enzyme solution, and freeze-dried once more. This dialysed crude extract was

resuspended in 0.1 M sodium acetate buffer (pH 5) again. Thus, the enzyme concentration in solution was increased 500-fold. It was analysed by SDS-PAGE (0.1% SDS, 12% polyacryalamide). 50 µl of enzyme solution was added to 50 µl dissociation buffer (0.5 M Tris, pH 6.8; glycerol; 10% SDS; β-mercaptoethanol; 1% bromophenol blue), boiled for 5 minutes and 20 µl of this solution was loaded onto the gel. A broad range molecular weight marker (Roche) was also run alongside the sample in order to determine the size of the laccase protein. The gel was run at 130 V for 2.5 hours and then stained in Coomassie Brilliant Blue solution (2.5 g Coomassie, 40% methanol, 7% glacial acetic acid and 53% $dH_2O$) overnight. To visualise the stained protein, the gel was placed in destain solution (40% methanol, 7% glacial acetic acid and 53% $dH_2O$) for 24 hours. A separate gel was run and stained in ABTS solution (5 mM) to confirm the presence of the laccase enzyme.

### 2.7.5 Reaction of free laccase with olive wastewater

The effect of free laccase on the total phenols content of olive wastewater was investigated at various concentrations of the olive wastewater by the addition of different amounts of enzyme. Olive wastewater samples (5 ml) diluted to 25, 50, 75% (v/v) and undiluted were incubated with 2 and 5 U of the crude laccase solution at 28°C. Samples (100 µl) were taken at 6 and 20 hours of incubation, for total phenols analysis and pH determination. HPLC analysis was conducted with 1 ml of sample after 20 hours incubation. Control experiments with $dH_2O$ added in place of enzyme solution were also conducted.

### 2.7.6 HPLC analysis

Reverse phase HPLC was conducted on samples from the reaction of laccase with olive wastewater. Monophenolics present in the olive wastewater were separated by isocratic elution using a mobile phase of 80:20:2.5 $H_2O$:Methanol:Acetic acid at a flow rate of 1ml/min. The same equipment was used as described in section 2.1.5.

# CHAPTER 3

# RESULTS AND DISCUSSION

In this chapter the results obtained from the investigations conducted for the biodegradation of olive wastewater are presented and discussed.

## 3.1 Characterisation of olive wastewater

The olive wastewater used in this study was obtained from tanks of black olive (Kalamata) fermentations at the Buffet Olive plant in the Western Cape (see section 2.1). Table 3.1 shows the results of chemical analysis of the wastewater.

**Table 3.1: Composition of the olive wastewater used in this study.**

| | |
|---|---|
| pH | 4.5 – 5.5 |
| Conductivity ($mS.cm^{-1}$) | 82 – 98 |
| COD ($g.L^{-1}$) | 55 – 82 |
| Total phenols ($g.L^{-1}$) | 1.78 – 5.28 |
| Nitrogen ($g.L^{-1}$) | 0.03 – 0.2 |
| Reducing sugar ($g.L^{-1}$) | 0.22 – 0.34 |
| Total solids ($g.L^{-1}$) | 92.1 – 139 |
| Dissolved solids ($g.L^{-1}$) | 91.2 – 137.9 |
| Suspended solids ($g.L^{-1}$) | 0.3 – 1.07 |
| TOC ($g.L^{-1}$) | 6.25 – 18.35 |
| Lipids ($g.L^{-1}$) | 0.56 – 1.83 |

Olive wastewaters, both olive mill wastewater (OMW) and olive fermentation brines (OFW), are acidic due to the presence of phenolic and other organic acids (e.g. lactic, acetic, elenolic acids). Wastewaters from the production of green olives have high pH values, due to the sodium hydroxide used in the debittering (lye) treatments. Lye treatments are used to hydrolyse oleuropein, the compound responsible for the natural bitterness of olives (de Castro and Brenes, 2001). Since black olives are picked at an advanced stage of maturity, they have little oleuropein content because the majority of the oleuropein content has been hydrolysed to hydroxytyrosol (Romero *et al.*, 2004). For this reason, black olives do not require lye treatment. As a result of this, wastewaters from the black olive processing industry are consistently acidic. They also have a high

salt content (measured as conductivity) due to the salt required for the brining process. This poses another problem in the bioremediation of these wastewaters because highly saline environments are deleterious to the growth of many plants and microorganisms.

Chemical oxygen demand (COD) can be used as a measure of the pollutants in wastewaters and fresh waters. COD is defined as the amount of specified oxidant that reacts with a sample under controlled conditions. Most types of organic matter are oxidised by a boiling mixture of chromic and sulphuric acids, as occurs in the standard method for COD measurement (APHA Standard methods, 1998). The wastewaters investigated in this study have high COD values (Table 3.1) and therefore must be remediated prior to disposal if they are to be disposed of in municipal waterworks. The total phenolics concentration can be as high as 5 g/L and it is these compounds that pose the environmental threat. The simple monophenols have phytotoxic and antimicrobial effects. The two predominant simple phenols present in this wastewater are hydroxytyrosol (3,4-dihydroxyphenyl ethanol) and tyrosol (4-hydroxyphenyl ethanol) (Fig. 1.5). These two compounds comprise approximately 35 – 50% of the total phenols concentration in black olive fermentation brines. Their peaks were identified using pure hydroxytyrosol and tyrosol as model compounds (Fig. 3.1).

The olive wastewater is low in nitrogen content (0.2 g/L). The COD to nitrogen ratio of this wastewater is approximately 595:1 and a ratio of 20:1 is desirable to maintain satisfactory microbial activity, in order to achieve biological degradation (Benitez *et al.,* 1997). From this it was assumed that the olive wastewater would need to be supplemented with a source of nitrogen in order to stimulate microbial growth, to facilitate degradation of the initial organic load and the phenol content of the wastewater. Compared to OMW, olive brine wastewaters have relatively low sugar and lipid content. This is because there is little cell disruption during the brining process, whereas OMW contains olive pulp which includes cellular constituents, as a result of the milling process. Any sugars that are present in the brining waters would be readily assimilated and rapidly used up by the fermenting microorganisms during the brining process. This low sugar content of the olive wastewaters makes their biological degradation more difficult

because the phenolic compounds are the only available carbon source. Sayadi and Ellouz (1992) suggested that phenol removal from OMW with a strain of *Phanerochaete chrysosporium* occurred either in primary or secondary growth, depending on the carbon source which was added. Often, xenobiotic pollutants or compounds that are not readily utilised can only be degraded if a primary carbon source is available, a phenomenon known as co-metabolism (Madigan *et al.,* 2000). This illustrates the difficulty experienced when attempting the bioremediation of olive fermentation wastewaters because the primary carbon source available are the phenolic compounds.

**a)** **b)**

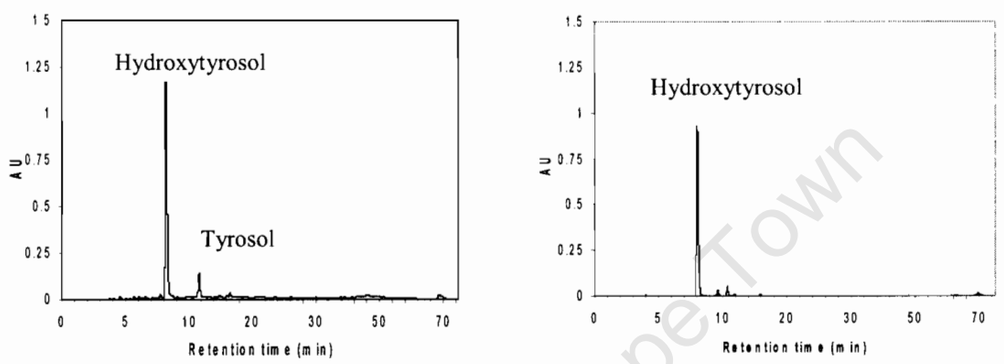



**Figure 3.1: HPLC chromatograms showing the two major phenolic compounds, hydroxytyrosol and tyrosol, present in olive wastewater (a) and in an ethyl acetate extract of the olive wastewater (b).**



## 3.2 Isolation and screening of microorganisms capable of growth on olive wastewater media

The 35 strains isolated from the evaporation ponds at the Buffet Olives plant (by A. van Schalkwyk) were cultured in broth or on agar plates containing 25% (v/v) olive wastewater and 5 g/L yeast extract (YE). Since these strains were isolated from the evaporation ponds, they are able to survive the toxic environment of this wastewater.

This ability makes these strains interesting from a bioremediation perspective because they are able to survive in an environment with low levels of readily utilisable carbon sources and must therefore have the ability to metabolise the phenolic compounds present in the olive wastewater. For this reason it was important to study their degradation capabilities. Once all of the strains had been isolated, it was necessary to select, from them, the organisms that would perform best in the bioremediation of this particular waste. Since the organisms had not yet been identified and it was possible that many of them may well have been replicates, a preliminary screening procedure, based solely on their ability to grow on the solid medium containing olive wastewater at 25% (v/v), was used to select for the most promising isolates.

From the growth experiments using 25% (v/v) olive wastewater and YE agar media, organisms AS-1, AS-3, AS-9, AS-12, AS-28 and AS-35 were selected. These isolates showed the best growth with colony numbers exceeding 15 on each of the plates. These counts were higher than any other organisms that were screened. These strains all appeared to have a very similar morphology. The strong growth shown by these organisms indicated that they were able to utilise the constituents in the olive wastewater as a source of carbon, and therefore it was decided that these strains would be used for preliminary degradation studies.



### 3.3 Initial studies for the degradation of olive wastewater by selected isolates

Strains AS-1, AS-3, AS-9, AS-12, AS-28 and AS-35 were investigated for their abilities to reduce the total phenol content of 25% (v/v) olive wastewater. The strains all showed similar degradation ability in terms of total phenols removal (Fig 3.2). At a 25% (v/v) dilution, the total phenolic content of this wastewater had an initial value of approximately 870 $mg.L^{-1}$ and after 144 hours of fermentation, the candidate organisms were each able to reduce the level to approximately 320 $mg.L^{-1}$. Uninoculated control experiments were also conducted. These controls showed no reduction in total phenols which indicates that no chemical oxidation was occurring. The maximum degradation rates, with respect to total phenols degradation, were determined as follows:

$\Delta S/\Delta t$ = Max. degradation rate ($g.L^{-1}/day$)

$\Delta S$ = Difference in total phenols concentration (g/L)

$\Delta t$ = Corresponding time period for the degradation (days)

Strains AS-1 and AS-3 showed slightly higher maximum rates of degradation, determined between 24 and 48 hours, of 0.33 and 0.362 $g.L^{-1}/day$ respectively, as compared to the other strains investigated. Strains AS-28 and AS-35 showed maximum degradation rates in the first 24 hours of fermentation. Strains AS-12, AS-28 and AS-35 showed the highest overall percentage removals of total phenols: 66, 68 and 65% respectively.

After similar biodegradation studies on the olive wastewater were conducted by our collaborators (the ARCAM research group at UWC) strain AS-35 was selected by the project team, based on the observation of new, unidentified peaks that appeared in the HPLC analyses from samples of the fermentation of olive wastewater with AS-35 (A. van Schalkwyk, unpublished results). On the basis of the growth of strain AS-35 on solid olive wastewater media and the observation of a range of products on HPLC analysis, this strain was selected for use in a comparative study of the locally isolated organism and a commercially available fungal strain. The comparison would be made on the basis of effective biodegradation of organics in olive wastewater.

AS-35 was shown to have the greatest sequence homology with *Bacillus megaterium* after 16S rRNA gene analysis (see section 2.5.1) (A. van Schalkwyk, unpublished results). From this point on strain AS-35 was known as *B. megaterium* AS-35.

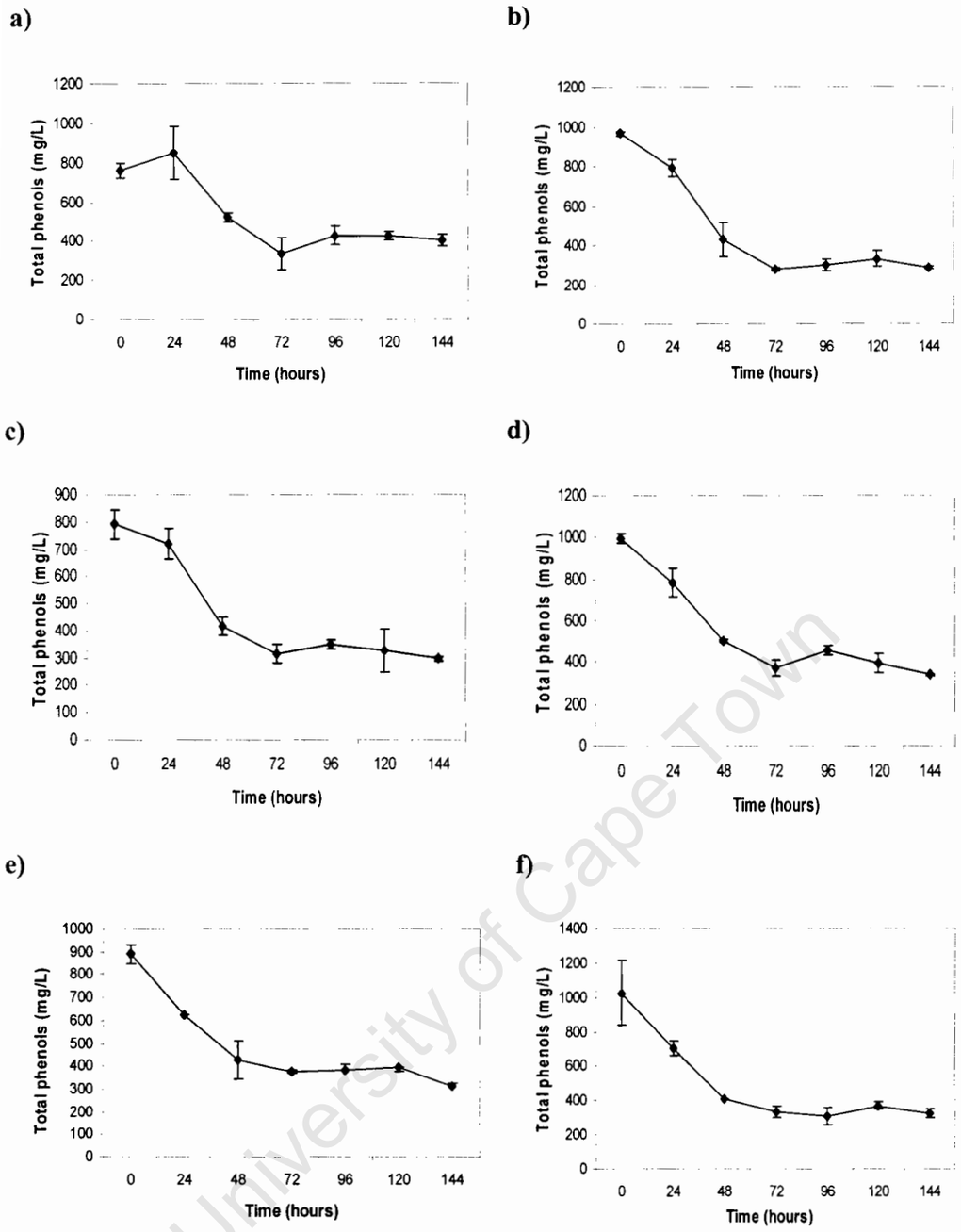





**Figure 3.2: Total phenols removal from 25% (v/v) olive wastewater by isolates from the evaporation ponds, AS-1 (a); AS-3 (b); AS-9 (c); AS-12 (d); AS-28 (e); AS-35 (f).**

## 3.4 Olive wastewater degradation by *B. megaterium* AS-35 in a stirred tank reactor

Small scale degradation studies conducted in shake flask culture are simple and allow screening procedures to be carried out to determine the potential of a selected microorganism for degradation of organics. They do not, however, allow for control of fermentation parameters such as temperature, pH or dissolved oxygen. The use of a controlled fermenter allows for complete control and constant monitoring of fermentation parameters, such as pH, dissolved oxygen and temperature. The ultimate aim of this investigation would be the development of a full scale remediation process for the treatment of large volumes of olive wastewater. To this end, studies were conducted with larger volumes (5L) in a controlled fermenter (Fig. 2.1), allowing a determination of the effects of pH on the degradation achieved by *B. megaterium* AS-35.

### 3.4.1 Fermentation of *B. megaterium* AS-35 using 25% (v/v) olive wastewater in the BioFlo 110 fermenter

The fermentation of *B. megaterium* AS-35 was conducted in a controlled fermenter with 5L of 25% (v/v) olive wastewater, at 28°C, monitoring the pH for the duration of the fermentation. The *B. megaterium* AS-35 culture was able to reduce the total phenol concentration from an initial value of 850 mg/L to 369 mg/L over a period of 72 hours (Fig. 3.3). The overall total phenols removal (57%) is slightly less than was achieved in shake flask culture (65%) but the maximum degradation rate was improved from 0.265 $g.L^{-1}$/day in shake flask culture to 0.481 $g.L^{-1}$/day in the fermenter. The improved maximum degradation rate, which occurred between 24 and 48 hours in the stirred tank as compared to the first 24 hours in the shake flasks was, most likely, due to better oxygen mass transfer that is achieved in stirred reactors compared to flasks. It was determined that 20.58% of total phenols were removed per gram of biomass. The total phenols removal reached a plateau after 48 hours in the shake flask and stirred tank reactor experiments.

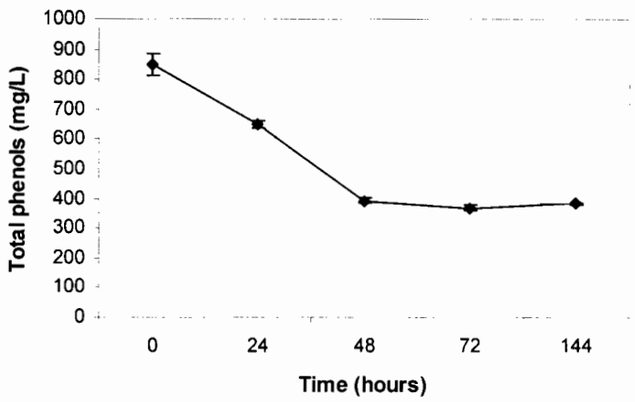



**Figure 3.3: Total phenols removal from 25% (v/v) olive wastewater by *B. megaterium* AS-35 in the BioFlo 110 fermenter.**

In the first 24 hours of fermentation in the fermenter the pH increased rapidly from 4.6 to pH 8. The maximum pH of 8.9 was reached after just 79 hours of fermentation and the pH remained at this value until the end of the experiment (Fig. 3.4). It is possible that the increase in pH is due to the consumption of organic acids present in the olive wastewater as a carbon source, to promote growth of the organism. This has been reported previously by Assas *et al.* (2002) with *Geotrichum candidum* in the fermentation of OMW. The pH curve has a sigmoidal shape with obvious lag, log and stationary phases and the growth curve is parallel with it (Fig. 3.4). During exponential growth, an increase of more than 1 pH unit was observed in less than 3 hours.

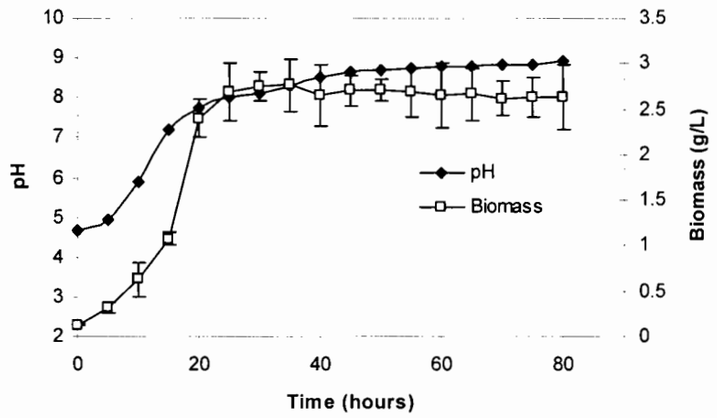



**Figure 3.4: pH profile and growth curve of the fermentation of *B. megaterium* AS-35 in 25% (v/v) olive wastewater in the BioFlo 110 fermenter.**

This rapid increase in pH was accompanied by a significant darkening of the waste in the first 24 hours that was not observed in uninoculated control experiments. This darkening of the wastewater suggests polymerisation of phenolic compounds into larger polymers that are darker in colour. Similar results have been reported by other researchers and these reports have attributed the polymerisation to an increase in pH. Assas *et al.* (2002) reported that the consumption of organic acids by *G. candidum* in stored OMW resulted in an increase in pH and led to increased autooxidation of phenolic compounds into compounds with higher molecular weight and resistance to biodegradation. Qin *et al.* (1997) noticed that the colour of green tea catechins changed from light to dark brown with an increase in pH, and the same effect was reported by Field and Lettinga (1991) with *A. niger* on spruce bark extracts, when tannins were autooxidised and polymerised at a pH in excess of 6 to give recalcitrant, dark polymers. Ayed and Hamdi (2003) reported that decolourisation of OMW was improved by maintenance of a low pH.

An increase in pH has also been reported to be a result of the formation of larger polycondensed structures. Hafidi *et al.* (2005) stated that an increase in pH, during aerobic fermentation of OMW, may be due to the integration of compounds with COOH and OH groups through polycondensation during the formation of humic structures. It has

also been shown that simple phenolic compounds polymerise during storage of OMW and reach high molecular weights (Hamdi, 1992). The increase in colour observed in our study suggests that the increase in pH initiated a polymerisation process or autooxidation, which resulted in pigmented polyphenolic compounds. However, the maximum degradation rate of total phenols occurred between 24 and 48 hours, after the pH had already risen sharply and the colour of the wastewater had darkened. A possible explanation for the observed removal of phenols is that during these initial polymerisation processes, the polyphenolic compounds responsible for the colour of the wastewater combine to form larger, more darkly coloured compounds. There may still be sufficient monomeric phenols that the organism is able to utilise as a carbon source which results in a reduction of total phenols concentrations. In addition to this, the formation of polycondensed structures also leads to a decrease in total phenols measured (Hafidi *et al.,* 2005). The increased pH, darkening of the waste and reductions of total phenols were not observed in uninoculated control experiments, confirming that these are not chemical oxidation processes.

It was observed in the fermentation experiment, as well as in flask degradation studies, that the degradation of phenols reached a plateau. In this particular case, the plateau was reached at the same time that pH of the medium reached a maximum. The reason for this may be that the larger polyphenolic structures formed are stable, and are resistant to biodegradation (Assas *et al.,* 2002; Sayadi *et al.,* 2000; Hafidi *et al.*, 2005). This process is known as humification and the resulting humic acid-like compounds are resistant to biodegradation due to their reduced bioavailability (Hafidi *et al.,* 2005). Therefore complete removal of phenolic compounds is unlikely to be achievable. The process of humification is not an altogether negative result of an aerobic treatment, since it has been suggested, in numerous studies, to be a good index of stability and for assessing the agronomic value of organic residues to be used for agricultural composting (Hafidi *et al.,* 2005; Tomati *et al.,* 2000; Ouatmane *et al.,* 2000; Senesi *et al.,*1996). Thus, at the end of this aerobic treatment the resulting wastewater has a high concentration of large humic acid-like compounds which can be useful if it is to be used for composting. Unfortunately, the high salt concentration of this particular wastewater prevents it from

being used as compost due to the possibility of soil salinisation, which would be detrimental to the microflora of the soil to which the wastewater was applied (see section 1.8.1). Therefore, a future study towards the removal of the salt from the treated wastewater would enable the beneficial utilisation of the wastewater for agricultural purposes.

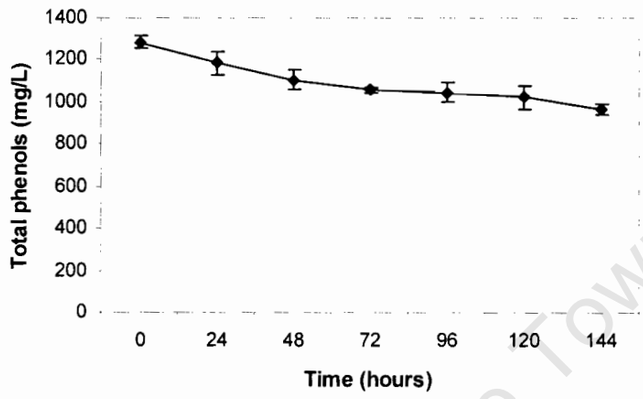
### 3.4.2 Fermentation of *B. megaterium* AS-35 in 25% (v/v) olive wastewater in the BioFlo 110 at constant pH

The darkening of the olive wastewater during fermentation indicated polymerisation of the phenolic compounds, resulting in polymeric structures resistant to biodegradation. Therefore, it was considered that if the pH was maintained at a constant value, the autoxidation processes that result in dark polymers might be reduced and the organism might be able to remove a larger phenolic fraction, with a reduction in the colour of the waste. Fermentations were conducted in the BioFlo 110 fermenter with 25% (v/v) olive wastewater (5 g/L YE) at controlled pH values of 6 and 7. The pH of the medium was adjusted to 6 or 7, prior to sterilisation, with 1M NaOH and these pHs were maintained by the addition of 0.5M HCl. Results shown in figure 3.5 indicate that constant pH was detrimental to the removal of phenolics in the fermentation. At pH 6, only 20% of the phenols were removed from solution, from 1282 mg/L to 1020 mg/L, over a period of 144 hours. A similar result was obtained with the fermentation at pH 7, where the phenols were reduced from 1003 mg/L to 775 mg/L, a reduction of only 22%. Similar results were obtained by our collaborators, who found that the total phenols removal with *B. megaterium* AS-35 was lower at pH 6 and pH 8 compared to that achieved when the pH of the medium was not controlled (A. van Schalkwyk, unpublished results).

Medici *et al.* (1985) showed that at neutralised or slightly alkaline pH, the phenol moieties are changed to phenates and their antimicrobial character is reduced, allowing increased microbial activity. Although the reduction in total phenols concentration in the above pH controlled experiments was much lower than was achieved without maintaining the pH, there did seem to be significant microbial activity, as indicated by

the large volumes (exceeding 1L) of HCl required to maintain the low pH of the fermentation medium. This is confirmed by the fact that uninoculated control experiments showed no increase in pH. The fact that maintaining a constant low pH was detrimental to the biodegradation of the olive wastewater was an important result because a treatment process that does not require costly additions, such as pH maintenance, would be a more feasible financial option in developing a full scale treatment process.

**a)**




**b)**

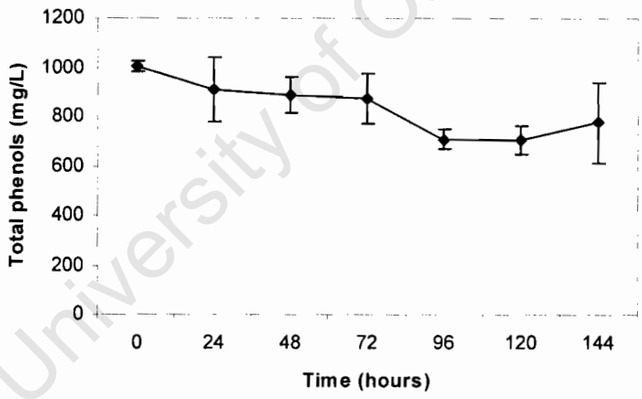





**Figure 3.5: Total phenols removal by *B. megaterium* AS-35 in 25% (v/v) olive wastewater at pH 6 (a) and pH 7 (b) respectively.**

## 3.5 Comparison of olive wastewater degradation and decolourisation capabilities of *B. megaterium* AS-35 and *T. pubescens*

The use of single organisms is an effective way to investigate the biodegradability of a complex waste for the design of a potential bioremediation process because it allows for uncomplicated re-inoculation from stock cultures and allows for better prediction of the fate of the compounds present in the wastewater. This investigation was conducted in order to better understand the growth and degradation dynamics that take place during the bioremediation of olive wastewater with two different organisms. A comparison of different microbial systems could elucidate which type of organism would best be suited for the remediation of the complex olive wastewater. Bacteria and fungi are different with respect to growth rates and metabolism of different carbon sources including lignin and aromatic compounds. Both groups are known to produce extracellular enzymes capable of degrading lignocellulose in a process of concerted oxidative and reductive conversions (Tuor *et al.,* 1995), but more research has been done with white-rot fungi for the production of these industrially important enzymes (Table 3.2). Laccases that have been produced and used for bioremediation of synthetic dyes (Knutson and Ragauskas, 2004; Zille *et al.,* 2005; Robinson *et al.,* 2001) and chlorophenolic compounds, from the bleaching of pulp (Roy-Arcand and Archibald, 1991), were isolated from various white-rot fungi.

The benefits of investigations conducted using a CSTR have already been discussed (see section 3.4), but it is not the only reactor configuration of interest for bioremediation studies. Pneumatic reactors, in which all the agitation is achieved by bubbling gas, are a more recent type of reactor system. Airlift reactors are one type of pneumatic reactor consisting of a liquid pool that is divided into two distinct zones, only one of which is sparged by gas (Chisti and Moo-Young, 1987). The benefits of this type of reactor configuration are their ease of design and construction, low power input to achieve the required transport rates and low shear fields. Such reactors are of particular interest for working with fungi, which are known not to tolerate high shear stresses. Airlift reactors are also ideal for pure culture studies due to their potential for extended aseptic operation,

which arises as a result of the elimination of stirrer shafts, seals and bearings (Chisti and Moo-Young, 1987). The airlift reactor used in this study is illustrated in figure 2.3.

### 3.5.1 Investigation of the biodegradation of 10% (v/v) olive wastewater with *B. megaterium* AS-35

The previous degradation studies conducted with isolate *B. megaterium* AS-35 were conducted in 25% (v/v) olive wastewater. In this investigation, which served as a comparison of the biodegradation capabilities of a bacterium and a fungus, a 10% (v/v) dilution of olive wastewater was used in order to minimise factors such as salinity, which may serve to inhibit the growth of the organisms and therefore give a more realistic comparison of their biodegradation capabilities.

The maximum specific growth rates ($\mu_{max}$) of *B. megaterium* AS-35 on 10% (v/v) olive wastewater were determined (section 2.5.5) to be 0.105, 0.122 and 0.164 $h^{-1}$ for the shake flask, airlift and stirred tank reactors respectively (Table 3.2). The levels of total phenols removed (Table 3.2) that were observed in the previous experiments (see section 3.4) were not reproduced in the 10% (v/v) dilution of the waste and a 45% reduction of the total phenolic fraction was achieved in shake flask culture (Fig. 3.6). A significantly longer lag phase was observed for the stirred tank reactor (Fig 3.8). The reduction of phenols concentration achieved was 25% in both the airlift and stirred tank reactors (Figs. 3.7 and 3.8). The amount of phenols (g) removed per gram of biomass were determined to be 0.12, 0.07 and 0.04 for the shake flask, airlift reactor and stirred tank reactor, respectively (Table 3.2). This indicates that *B. megaterium* AS-35 was most efficient at removing phenols in shake flask culture compared to the airlift and stirred tank reactors.

**Table 3.2: Comparison of growth parameters and total phenols removed from 10% (v/v) olive wastewater obtained with strain *B. megaterium* AS-35 in different reactors.**

| | Reactor | | |
|---|---|---|---|
| **Parameter** | **Shake flask** | **Airlift** | **CSTR** |
| Max. dry weight (g/L) | 1.39 | 3.14 | 2.845 |
| $\mu_{max}$ ($h^{-1}$) | 0.105 | 0.122 | 0.164 |
| Total phenols (% removal) | 45 | 25 | 25 |
| Max. degradation rate ($g.L^{-1}$/day) | 0.096 | 0.051 | 0.112 |
| g phenols removed/ g biomass | 0.12 | 0.07 | 0.04 |
| %COD removed | 47 | 32 | 35 |

The maximum phenols degradation rates (dS/dt) were determined between 24 and 48 hours for the shake flask and stirred tank reactor experiments and between 72 and 96 hours for the airlift reactor experiment. These were determined to be 0.096, 0.051 and 0.112 $g.L^{-1}$/ day for the shake flask, airlift and stirred tank reactor, respectively (Table 3.2) and were significantly lower than obtained previously (see sections 3.3 and 3.4.1). One possible reason for the reduced degradation efficiency in the experiments conducted in 10% (v/v) olive wastewater could be the altered medium on which the strain was cultured. Originally, the strain had been cultured on 25% (v/v) olive wastewater agar plates, but this was later changed to a more nutrient rich medium comprising readily utilisable carbon sources, such as glucose, in order to obtain more biomass. The importance of this is that the production of enzymes required for the metabolism of alternative carbon sources, such as the phenolic compounds, would have been induced when this strain was cultured on the olive wastewater medium. These enzymes include phenol hydroxylase and catechol dioxygenases (see section 1.5.1). This phenomenon, induction, leads to the synthesis of an enzyme only when its substrate is present and usually when more readily oxidised carbon sources are not (Madigan *et al.,* 2000). When the culture medium was changed and readily utilisable carbon sources, such as glucose, were made available the organism could have ceased to express the genes coding for the phenol metabolising enzymes. The importance of acclimatisation of an organism (or consortium of organisms) to a complex medium containing different carbon sources, such as are present in this waste, has previously been discussed in chapter 1. The strain *B. megaterium* AS-35 would previously have been well acclimatised to the 25% (v/v) olive

wastewater medium, but in these experiments it could have been in an altered metabolic state, which could result in reduced levels of phenols removal. Another possible explanation for this could be that there may not have been enough carbon source available for the organism to utilise.

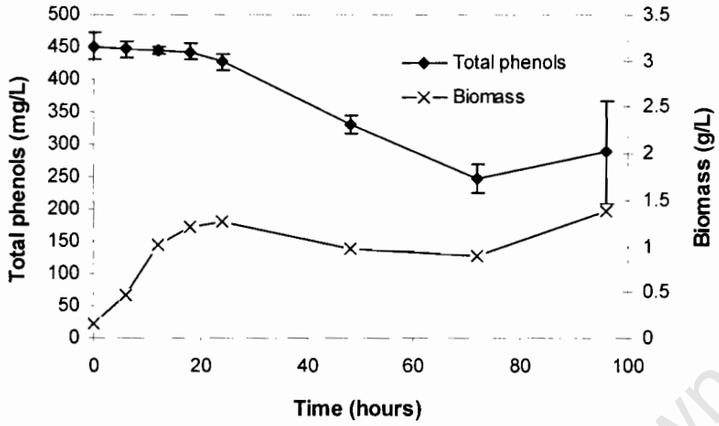



**Figure 3.6: Biomass growth and total phenols reduction in 10% (v/v) olive wastewater with *B. megaterium* AS-35 in shake flask culture.**

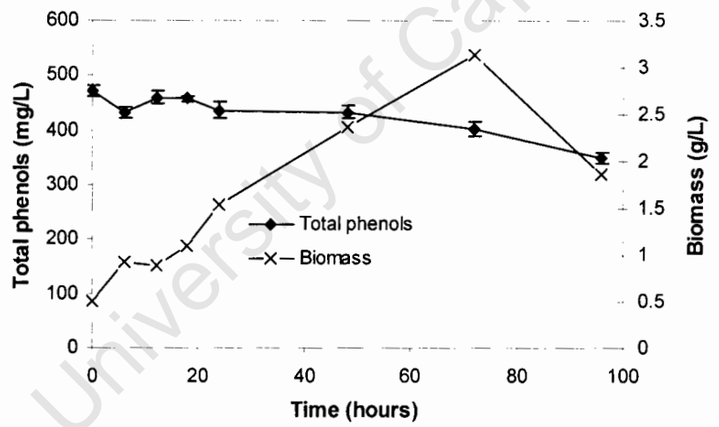



**Figure 3.7: Biomass growth and total phenols reduction in 10% (v/v) olive wastewater with *B. megaterium* AS-35 in the airlift reactor.**

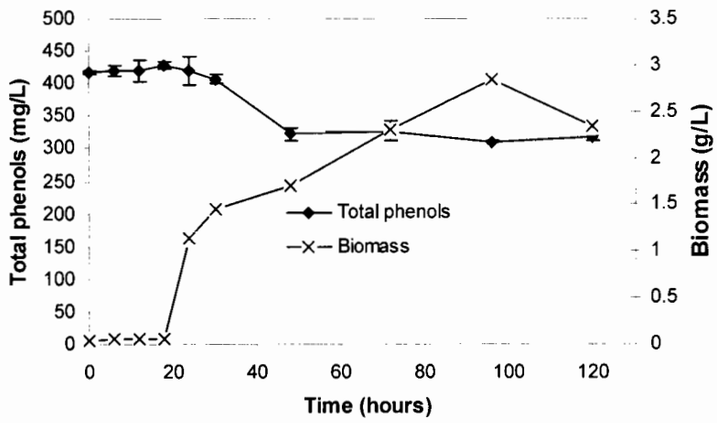



**Figure 3.8: Biomass growth and total phenols reduction in 10% (v/v) olive wastewater with *B. megaterium* AS-35 in the stirred tank reactor.**

In contrast to the low removal of phenols, the COD reductions observed using the 10% (v/v) olive wastewater, were similar to previous results. COD reductions of 32%, 35% and 47% for the airlift reactor, stirred tank reactor and shake flask indicate appreciable detoxification of the wastewater. This indicates that the organism utilised other compounds in the wastewater as a carbon source, such as the organic acids, which contributed to the COD determinations.

The colour of the fermentation media in the shake flasks, airlift and stirred tank reactors turned dark brown during the course of the experiments, just as was observed in the previous degradation studies with *B. megaterium* AS-35 (section 3.4). Again, this suggests the polymerisation of phenolic compounds into larger, more pigmented polymeric phenolic compounds. Figure 3.9 shows the colour variation of samples from the airlift reactor, indicating the significant increase in absorbance over the time of fermentation. Correlating with the darkening of the medium was the increase in pH (Fig 3.10). The most significant increases in colour and pH were observed between 18 hours and 48 hours. These increases in pH and colour were not observed in the uninoculated control experiments confirming, once more, that these are not chemical processes.

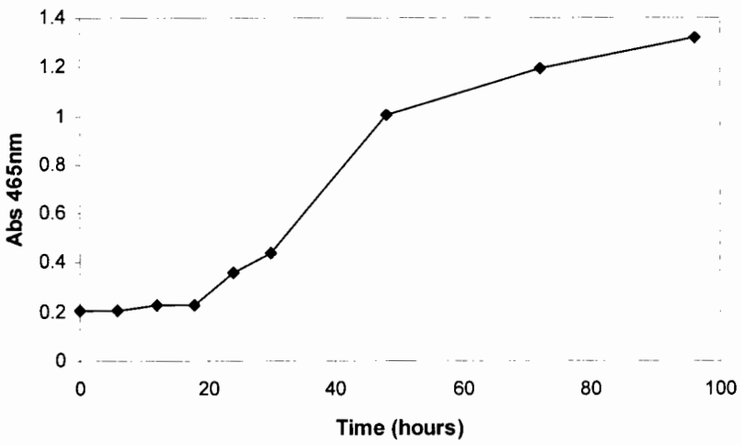



**Figure 3.9: Colour evolution during the fermentation of 10% (v/v) olive wastewater with *B. megaterium* AS-35 in the airlift reactor.**

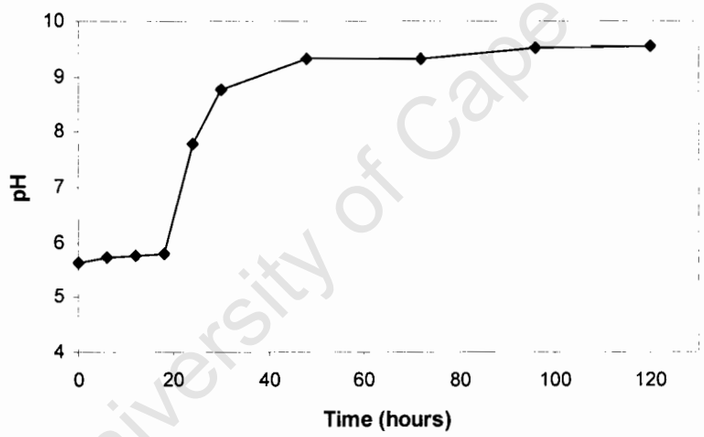



**Figure 3.10: The pH profile from the fermentation of 10% (v/v) olive wastewater with *B. megaterium* AS-35 in the stirred tank reactor.**

### 3.5.1.1 Determination of the fate of the phenolic compounds in olive wastewater by gel permeation chromatography

Samples from the airlift and stirred tank reactors, taken at the start and end of fermentations, were analysed by gel permeation chromatography on Sephadex G-50. This was done in order to qualitatively determine the impact of bacterial growth on the monomeric and polymeric aromatic fractions. Dextran blue (2000 kDa) and gallic acid (170 kDa) were used as standards. Dextran blue started to elute in fraction 14 and the maximum absorbance was reached in fraction 16 (Fig. 3.11). The void volume was thus determined to be 130 ml, since each fraction had a volume of 10 ml. A solution of humic acid (1g/L, Merck) was also run through the column as a comparison. Compounds from the humic acid sample eluted in the same fraction as Dextran blue indicating that compounds of this size are humic acid-like compounds.

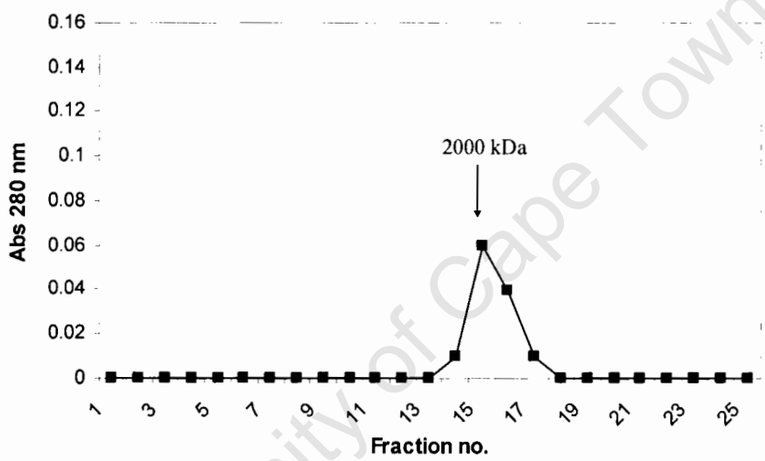



**Figure 3.11: Elution profile of Dextran blue (1 g/L) on Sephadex G-50 (70 x 2.5 cm, flow rate 1ml/min, 10 ml fractions collected).**

The molecular weight distribution of the phenolic fraction of olive wastewater, before fermentation, showed two major peaks (Figs. 3.12 and 3.13). The first corresponded to the largest, humic acid-like compounds with a molecular mass of approximately 2000 kDa or larger, eluting at approximately fraction 16. The second corresponded to a smaller fraction comprising medium and low molecular weight compounds, eluting in fractions

between 35 and 49, generally smaller than 8 kDa. Large polymeric compounds of high molecular weight are known to contribute to the colour of wastewaters generated by the olive industries (Sayadi *et al.,* 2000; Assas *et al.,* 2002), and chromophoric lignin-related materials with different degrees of polymerisation also contribute significantly to colour (Sayadi and Ellouz, 1992). The chromophoric lignin-related materials generally smaller than 8kDa were eluted after fraction 16 up to fraction 49 (Fig. 3.12 and 3.13).

When the sample components separated on the Sephadex column the absorbance readings of the fractions started to increase, indicating the elution of the largest phenolic compounds (~2000 kDa), but an intense brown/purple colour was still visible through the glass of the column. This indicated that the colour of the waste can be attributed to the large polyphenolic compounds as well as smaller compounds with varying degrees of polymerisation. The molecular weight measurements of the samples taken at the end of the fermentation showed the formation of a significant peak of approximately 2000 kDa. This peak would have been formed as a result of the polymerisation of phenolic compounds into complex, lignin-like structures with a high molecular weight. There was also an increase in compounds with varying molecular weights which were eluted between fractions 18 and 38. These compounds would have formed as a result of varying degrees of polymerisation. Of particular interest were those between fractions 31 and 38 which were thought to be condensed tannins because these compounds usually exist as oligomers, containing two to five or six flavan-3-ol oligomeric units (Fig. 1.3) (Haslam, 1996). This confirmed that the increase in concentration of compounds of this nature also contributes significantly to the observed increase in colour of the fermented olive wastewater.

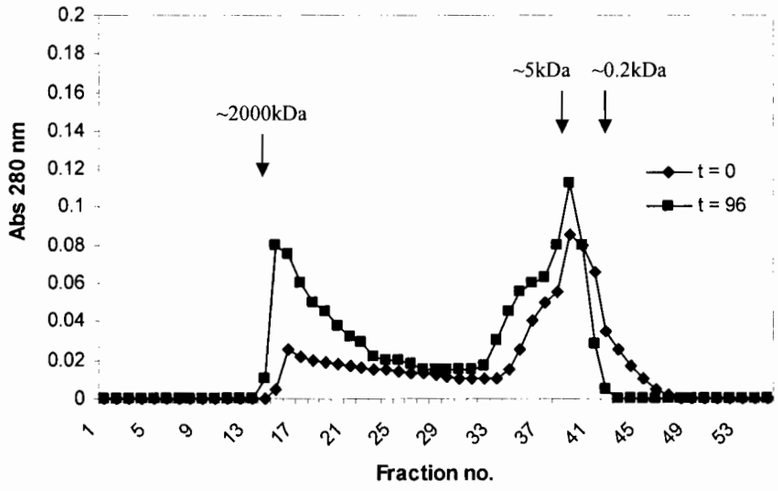



**Figure 3.12: Molecular weight distribution profiles of the aromatic fraction before and after fermentation of 10% (v/v) olive wastewater in airlift reactor culture with *B. megaterium* AS-35 (70 x 2.5 cm, Sephadex G-50, flow rate 1 ml/min, 10 ml fractions collected).**

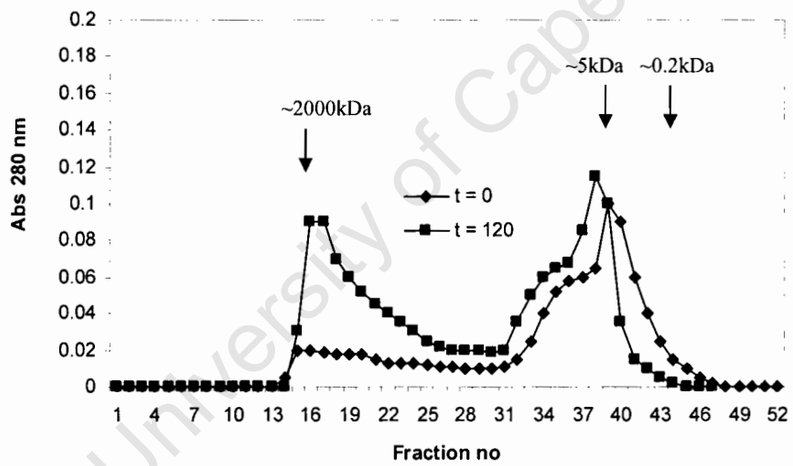



**Figure 3.13: Molecular weight distribution profiles of the aromatic compounds before and after the fermentation of 10% (v/v) olive wastewater in stirred tank culture with *B. megaterium* AS-35 (70 x 2.5 cm, Sephadex G-50, flow rate 1 ml/min, 10 ml fractions collected).**

### 3.5.2 Investigation of the biodegradation of 10% (v/v) olive wastewater with *T. pubescens*

Most of the aerobic degradation investigations that have been performed on olive wastewaters have involved the use of various fungi (Table 1.2), but, to our knowledge, this is the first investigation with *T. pubescens*. This fungus was used in work previously done in our research group on the bioremediation of stripped gas liquor, containing the monomers of phenol, *p*-, *m*- and *o*-cresol (Ryan *et al.*, 2005). *T. pubescens* is a basidiomyete with excellent laccase producing ability (Galhaup *et al.*, 2002). Basidiomycetes are chemo-organotrophic organisms and have the ability to utilise various organic carbon sources for their metabolism (Fell *et al.*, 2001). *T. pubescens* is widely recognised for its ability to produce laccase, and laccase is known to react with aromatic substrates in a non-specific manner (see section 1.6). Thus, it was decided to investigate this fungus for its ability to biodegrade the components of olive wastewater. As with *B. megaterium* AS-35, experiments with *T. pubescens* were conducted in 10% (v/v) olive wastewater in shake flask, airlift reactor and stirred tank reactor culture. Growth parameters, phenols removal and molecular weight distribution analyses were performed.

Bacteria have a faster doubling time than fungi and it would be expected that the fungus would require a longer fermentation time to achieve similar levels of phenols removal. From the data obtained, the growth of the fungus did not seem to be inhibited at this low concentration of olive waste and dry mass determinations of 3.12 g/L in shake flask culture were obtained, higher than that achieved by *B. megaterium* AS-35 (Table 3.3).

**Table 3.3: Comparison of growth parameters and total phenols removed from 10% (v/v) olive wastewater obtained with *T. pubescens* in different reactors.**

| | Reactor | | |
|---|---|---|---|
| **Parameter** | **Shake flask** | **Airlift** | **CSTR** |
| Max. mycelial dry weight (g/L) | 3.12 | 1.15 | 2.025 |
| $\mu_{max}$ ($h^{-1}$) | 0.034 | 0.042 | 0.029 |
| Total phenols (% removal) | 79 | 69 | 79 |
| Max. degradation rate ($g.L^{-1}$/day) | 0.098 | 0.085 | 0.191 |
| g phenols removed/ g biomass | 0.06 | 0.14 | 0.14 |
| %COD removed | 51.3 | 42 | 51.6 |

The maximum specific growth rates ($\mu_{max}$) of the fungus were determined to be 0.034, 0.042 and 0.029 $h^{-1}$ for the shake flask, airlift and stirred tank reactors respectively (Table 3.3). The increase in biomass observed suggests that the fungus was able to utilise the compounds in the olive wastewater as a source of carbon since the phenolic compounds are the major carbon source in the wastewater. A reduction in total phenols in excess of 70% was achieved, for each of the reactors with *T. pubescens* and maximum degradation rates, with respect to total phenols, were determined to be 0.098, 0.085 and 0.191 $g.L^{-1}$/day for the shake flask, airlift and stirred tank reactors, respectively. These maximum degradation rates were achieved in the first 24 hours in each of the reactors. The total phenols concentrations were reduced from 245 mg/L to 50 mg/L in the shake flask (79% reduction) (Fig. 3.14), from 233 mg/L to 72 mg/L in the airlift reactor (69%) (Fig. 3.15) and from 357 mg/L to 74 mg/L in the stirred tank reactor (79%) (Fig. 3.16). The amount of phenols removed (g) per gram of biomass produced was determined to be 0.06, 0.14 and 0.14 for the shake flask, airlift reactor and stirred tank reactor, respectively (Table 3.3). The values obtained for the airlift and stirred tank reactors were significantly higher than those achieved by *B. megaterium* AS-35 and indicate that the fungus was more efficient at phenolic removal from olive wastewater than the bacterium.

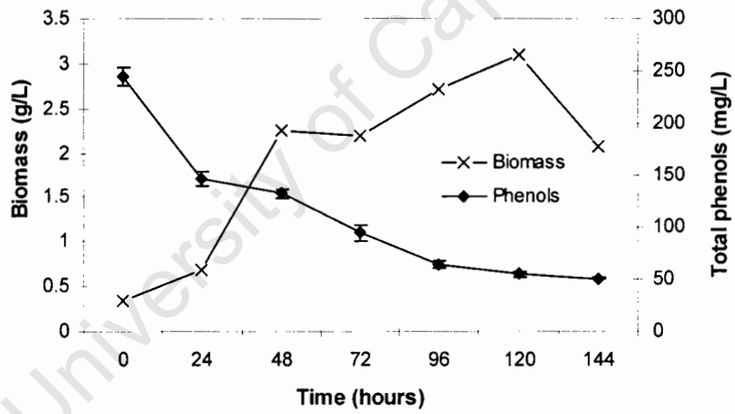



**Figure 3.14: Biomass production of *T. pubescens* and total phenols reduction in 10% (v/v) olive wastewater in shake flask culture.**

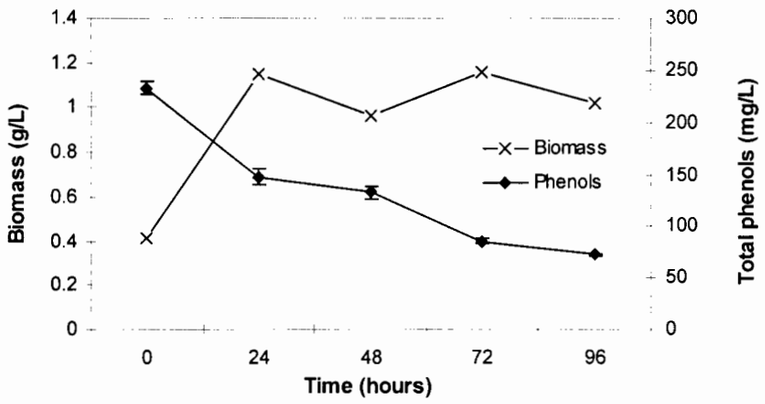



**Figure 3.15: Biomass production of *T. pubescens* and total phenols reduction in 10% (v/v) olive wastewater in the airlift reactor.**

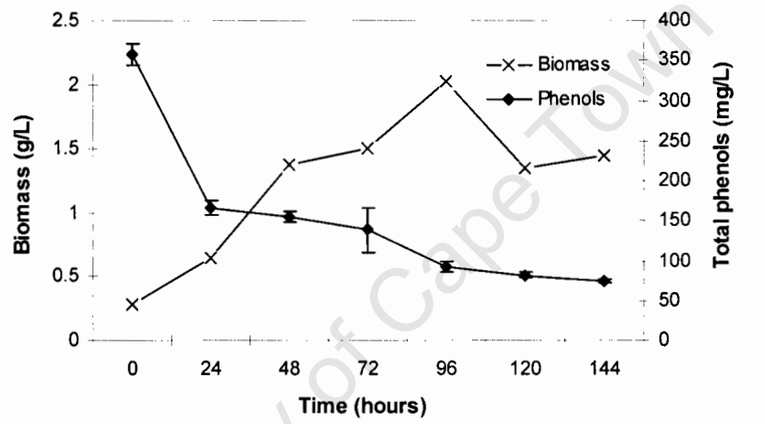



**Figure 3.16: Biomass production of *T. pubescens* and total phenols reduction in 10% (v/v) olive wastewater in the stirred tank reactor.**

### 3.5.2.1 Determination of the fate of the phenolic compounds in olive wastewater by gel permeation chromatography

As with samples from the fermentations with *B. megaterium* AS-35, samples taken at the start and the end of the fermentations with *T. pubescens* in 10% (v/v) olive wastewater were analysed by size exclusion chromatography on Sephadex G-50 (Figs. 3.17; 3.18; 3.19). Again, this was done to qualitatively determine the impact of fungal growth on the

monomeric and polymeric phenolic fractions of the wastewater. These investigations showed similar trends to those observed for *B. megaterium* AS-35 fermentations. There was a significant increase in the peak corresponding to approximately 2000 kDa, much the same as that observed in the molecular weight distributions of the bacterial fermentations. This can be attributed to the polymerisation of phenolic compounds into products of high molecular weight. More interesting is the reduction of the intermediate molecular weight fractions. Figures 3.17 and 3.18 showed similar distribution curves. However, the analysis of samples from the fermentation in the stirred tank reactor (Fig. 3.19), showed a slightly different trend, with a distinct decrease in compounds that elute between fractions 31 and 46. This was the only difference that could be observed between the data on molecular weight distributions from the bacterial and fungal fermentations. This suggests that the condensed tannins were broken down into smaller compounds that were less coloured.

It was stated earlier, in section 3.5.1.1, that compounds eluting between fractions 31 and 38 are thought to comprise condensed tannins and other phenolics with varying degrees of polymerisation. It is believed that they contribute to colour because the colour analyses of the samples (see section 2.5.10) from the fermentation of olive wastewater in the stirred tank reactor with *T. pubescens* (Fig. 3.20) indicated an overall decrease in colour intensity of approximately 10%. Other researchers have reported that the presence of sugars has a positive effect on colour removal by white-rot fungi (Sayadi and Ellouz, 1995; Dias *et al.,* 2004). This suggestion indicates that decolourisation of olive wastewater is more difficult since there are no sugars present in this olive wastewater. Therefore, further studies of the biodegradation of olive wastewater by means of co-metabolism with other readily utilisable carbon sources may provide a means to increase the level of decolourisation of olive wastewater that can be achieved with white-rot fungi.

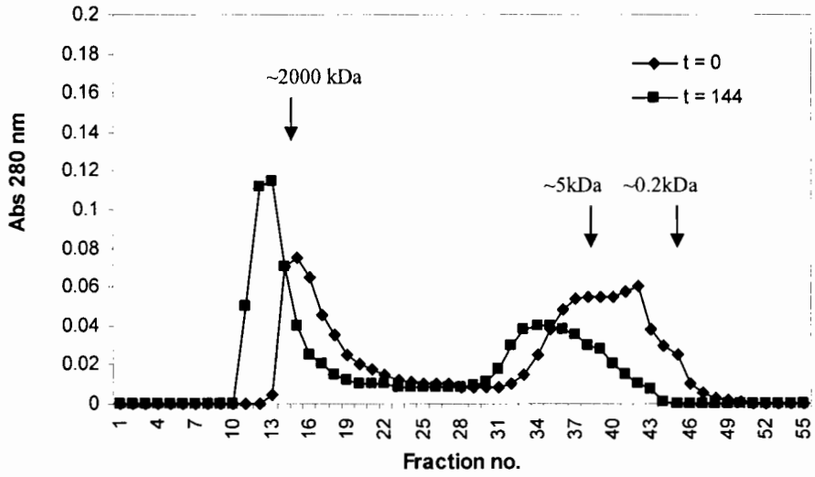



**Figure 3.17: Molecular weight distribution profiles of the aromatic compounds in olive wastewater before and after fermentation of *T. pubescens* in shake flask culture (70 x 2.5 cm, Sephadex G-50, flow rate 1 ml/min, 10 ml fractions collected).**

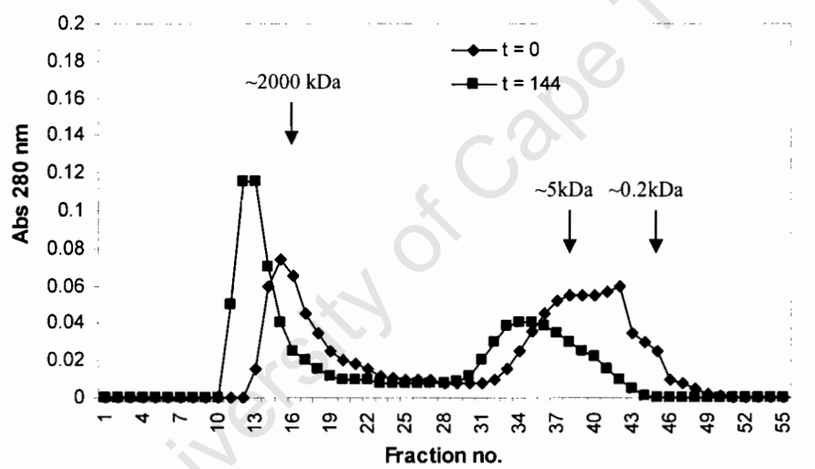



**Figure 3.18: Molecular weight distribution profiles of the aromatic compounds in olive wastewater before and after fermentation of *T. pubescens* in airlift culture (70 x 2.5 cm, Sephadex G-50, flow rate 1 ml/min, 10 ml fractions collected).**

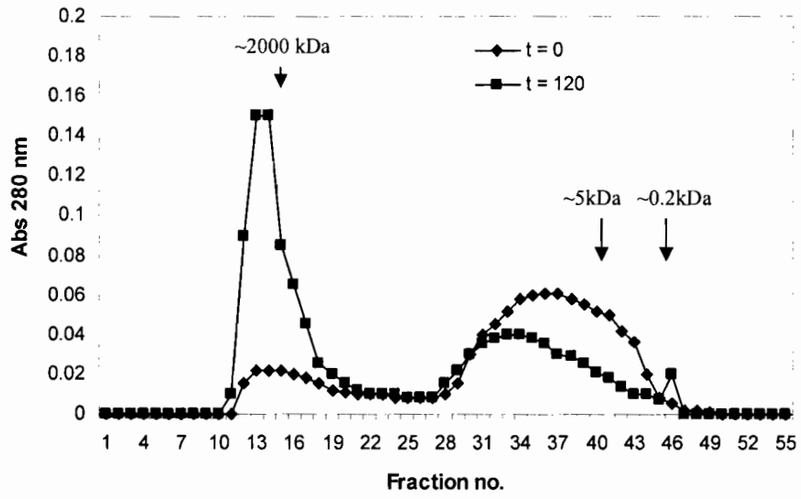



**Figure 3.19: Molecular weight distribution profiles of the aromatic compounds in olive wastewater before and after fermentation of *T. pubescens* in stirred tank culture (70 x 2.5 cm, Sephadex G-50, flow rate 1 ml/min, 10 ml fractions collected).**

The reduction in colour observed with *T. pubescens* fermentation of olive wastewater (Fig. 3.20) was thought to be due to the action of ligninolytic enzymes, and since this organism is a good laccase producer, laccase was initially expected to play a key role. The extent to which decolourisation can be attributed to the ligninolytic enzymes, and which of these may play the more crucial role, is a contentious issue among researchers. Perez *et al.* (1998) suggested that laccase may play a crucial role in the decolourisation of OMW. Some have reported the importance of LiP (Sayadi and Ellouz, 1995) but more recently there has been increased interest shown in the role that laccase may play (Perez *et al.,* 1998; Jaouani *et al.,* 2003; Dias *et al.,* 2004; D'Annibale *et al.,* 2004).

In this study, the pH determinations on samples from the fermentation of olive wastewater with *T. pubescens* showed that the pH remained below 6 throughout the fermentation. This low pH may limit the autooxidation of phenolics into larger polymers,

suggested to occur at alkaline pHs (Field and Lettinga, 1991; Bors *et al.,* 2000), and therefore it may be advantageous in allowing for decolourisation to occur. Assas *et al.* (2002) achieved significant levels of decolourisation of OMW with *G. candidum* where the pH remained between 4 and 5. Kim and Shoda (1999) found the same to be true for the decolourisation of spruce bark extracts with *A. niger.*

The mechanism employed by the fungus for maintaining this low pH is not clear but lignin degradation occurs at low pH (Tuor *et al.,* 1995) and the ligninolytic enzymes generally have their optima at lower pH. The pH optimum of laccase from *Phellinus noxius* was found to be 4.6 (Geiger *et al.,* 1986) and for *T. pubescens* it was found to be between 3 and 4.5, for phenolic substrates (Galhaup *et al.,* 2002).

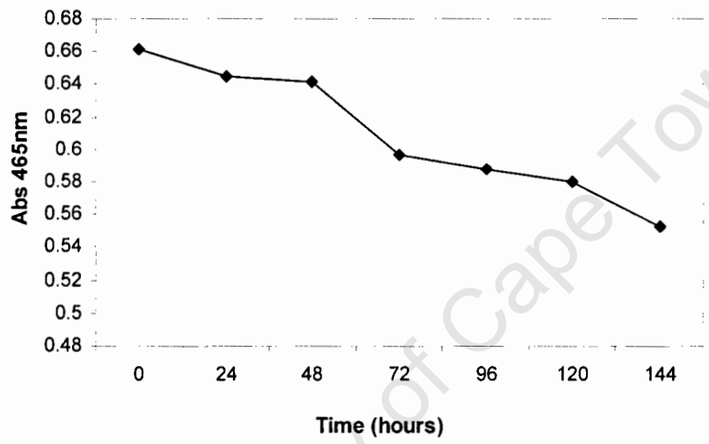



**Figure 3.20: Monitoring of the colour of samples from the fermentation of 10% (v/v) olive wastewater by *T. pubescens* in the stirred tank reactor.**

This study was aimed at finding the most suitable microorganism for biodegrading olive wastewater. The important criterion for measuring efficient biodegradation was the removal of phenolics. From the data presented on the fermentations of 10% (v/v) olive wastewater, *T. pubescens* removed a higher percentage of the total phenolics. Since the phenolic compounds are the predominant source of carbon in the olive wastewater and the fungus showed significant biomass production, it can be assumed that this fungus has

the ability to efficiently metabolise these phenolic compounds and hence reduce the toxicity of this wastewater. Therefore *T. pubescens* is a good candidate for a potential biological treatment process of olive wastewater. Coupled to the phenolics removal was a slight decolourisation of the wastewater which suggested the breakdown of chromophoric lignin-like compounds known to contribute to the dark colour of the waste. In view of these results, it was decided to conduct further investigations with *T. pubescens* and to investigate the role that laccase may play in the biodegradation of olive wastewater.

## 3.6 Further investigation of the biodegradation of olive wastewater by *T. pubescens*

### 3.6.1 Correlation of laccase production and growth of *T. pubescens* in 10% and 25% (v/v) olive wastewater

Experiments were conducted in order to determine the effect that increased concentration of olive wastewater could have on the growth and laccase production of *T. pubescens*, and to correlate these with removal of phenols from the wastewater. At an increased concentration of the wastewater (25% v/v), not only is there an increase in the organic load and phenolic fraction, already recognised to be toxic to microorganisms, but there is also an increase in salinity which would put the organism under significant osmotic stress. Experiments were conducted in 1L shake flasks with 10% and 25% (v/v) olive wastewater, in parallel. Experimental flasks were inoculated with 10% inoculum of actively growing *T. pubescens* cultured in TDM, from a four day old culture. Figures 3.21 and 3.22 show the growth and laccase production of *T. pubescens* grown in 10% and 25% (v/v) olive wastewater respectively. Laccase production reflected the trend of growth. This was particularly noticeable with cultures grown in 25% (v/v) olive wastewater, where a lag phase in the growth, of 48 hours, was observed, after which *T. pubescens* entered log phase and maximum biomass production was reached after just 72 hours. Cultures grown in 10% (v/v) olive wastewater showed growth within the first 24 hours, in contrast with cultures in 25% (v/v) olive wastewater. This indicates that the increased phenols concentration and salinity in 25% (v/v) olive wastewater slowed the growth of the fungus.

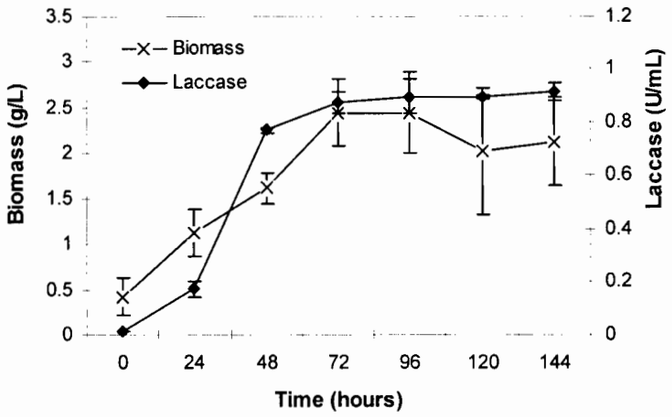



**Figure 3.21: Biomass growth and laccase production of *T. pubescens* fermentations in 10% (v/v) olive wastewater.**

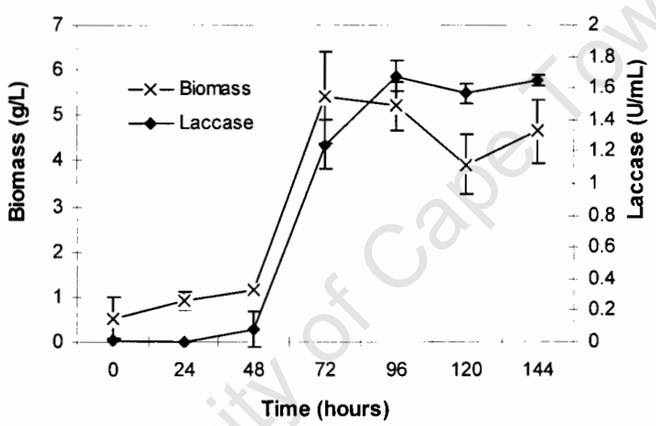



**Figure 3.22: Biomass growth and laccase production of *T. pubescens* fermentations in 25% (v/v) olive wastewater.**



Overall, *T. pubescens* biomass grew significantly better in 25% (v/v) olive wastewater, giving a final mycelial dry weight yield of 5.39 g/L. This can be compared with only 2.44 g/L biomass grown in 10% (v/v) olive wastewater after the same time. The maximum specific growth rate ($\mu_{max}$) achieved in 25% (v/v) olive wastewater was double that which was achieved in 10% (v/v) olive wastewater (Table 3.4). This could be attributed to the

increased amount of phenols available as a carbon source. A potential bioremediation process with *T. pubescens* would therefore only require a 25% dilution of olive wastewater, thereby reducing the cost of treatment. Future studies for the optimisation of the dilution factor may reduce this even further but it is expected that a threshold value would be reached due to the toxicity of the phenolic compounds.

Laccase production followed the growth trend and maximum laccase production was reached after 96 hours. There was no further increase in growth or laccase production after 96 hours. The laccase activities were found to be significantly higher in 25% (v/v) olive wastewater (reaching 1.6 U/mL) compared to 10% (v/v) olive wastewater where the maximum laccase activity was determined to be 0.9 U/mL (Table 3.4). However, the laccase production per gram of biomass was higher in 10% (v/v) olive wastewater (369 U/g) than in 25% (v/v) olive wastewater (297 U/g) (Table 3.4). This is not a significant difference though when considering the increased concentration of salt and phenols concentration of a 25% compared to a 10% (v/v) dilution of the olive wastewater.

**Table 3.4: Comparison of growth parameters, laccase production and removal of phenols from 10 and 25% (v/v) olive wastewater with *T. pubescens*.**

| Parameter | 10% OW | 25% OW |
|---|---|---|
| Mycelial dry weight (g/L) | 2.44 | 5.39 |
| $\mu_{max}$ ($h^{-1}$) | 0.017 | 0.036 |
| Laccase (U/mL) | 0.9 | 1.6 |
| Laccase (U/g biomass) | 369 | 297 |
| Total phenols (% removal) | 82.2 | 84.8 |
| Max. degradation rate ($g.L^{-1}$/day) | 0.164 | 0.318 |
| $E_{F/X}$ | 0.14 | 0.19 |
| g phenols removed/ g biomass | 0.1 | 0.14 |
| COD (% removal) | 52.5 | 53.6 |

OW = olive wastewater

The pH of the media was monitored throughout the fermentations (Fig. 3.23), and again it was noticed that in both cases the pH remained low, below 6, which is beneficial for the functioning of laccase.

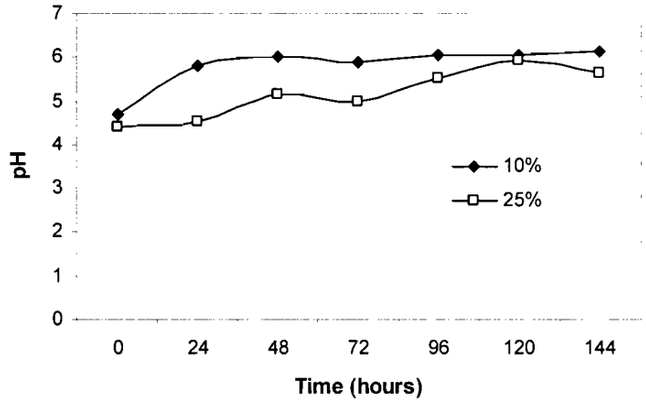



**Figure 3.23: pH profiles of the fermentations of 10% and 25% (v/v) olive wastewater with *T. pubescens*.**

The level of decolourisation achieved in the previous experiments (see section 3.5.2) was not achieved in these experiments. The absorbance values for the colour analysis showed a significant increase in the samples from the 25% (v/v) olive wastewater fermentation (Fig 3.25) than in those from the 10% (v/v) olive wastewater fermentation (Fig. 3.24). The most notable increases in absorbance were seen at the times when the highest production of laccase occurred, which would suggest polymerisation of the phenolic compounds due to the action of laccase.

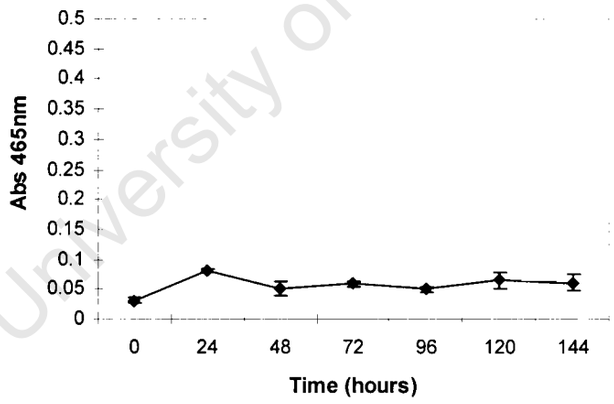



**Figure 3.24: Colour analysis of samples from the fermentation of 10% (v/v) olive wastewater with *T. pubescens*.**

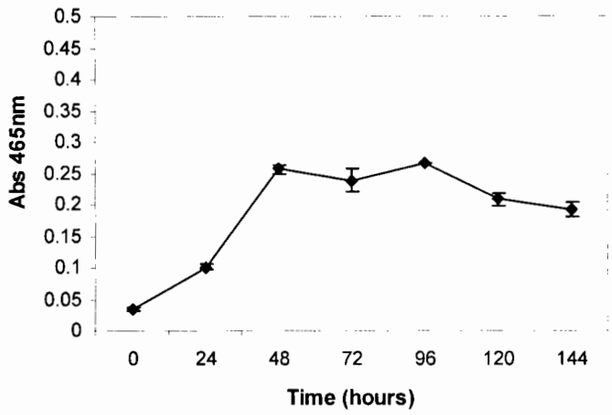



**Figure 3.25: Colour analysis of samples from the fermentation of 25% (v/v) olive wastewater with *T. pubescens*.**

### 3.6.2 Correlation of phenols removal and biomass production of *T. pubescens* in 10% and 25% (v/v) olive wastewater

Figs. 3.26 and 3.27 show the total phenolic removal compared to biomass production in 10% and 25% (v/v) olive wastewater, respectively. The removal of phenols and increase in biomass indicated that the phenols removal was related to the growth of the organism. In 10% (v/v) olive wastewater, 54% of the total phenols were removed in the first 24 hours, and the fungus showed significant growth in this period. In 25% (v/v) olive wastewater, only 19% of the total phenols were removed after the first 24 hours and this can be explained by the lag phase in growth experienced due to the increased concentration of olive wastewater, but after 48 hours, 55% removal had been achieved (Table 3.5).

**Table 3.5: The amount of phenols removed during the course of fermentation with *T. pubescens* in olive wastewater.**

OW = olive wastewater

| | Phenols removed (mg/L) | |
|---|---|---|
| **Time (hours)** | **10% OW** | **25% OW** |
| 24 | 165 (54%) | 164 (19%) |
| 48 | 217 (72%) | 482 (55%) |
| 72 | 241 (80%) | 633 (72%) |
| 96 | 249 (82%) | 726 (82%) |
| 120 | 249 (82%) | 739 (84%) |

Overall, phenols were reduced significantly by 82% in 10% (v/v) olive wastewater. In 25% (v/v) olive wastewater, they were reduced by 84%. This amounts to 0.1 g phenol/g biomass for 10% (v/v) and 0.14 g phenol/g biomass for 25% (v/v) olive wastewater (Table 3.4). These values indicate that *T. pubescens* was more efficient at metabolising phenols in the higher concentration of the wastewater. These levels of reduction of phenols were higher than any of the reductions achieved in fermentations with *B. megaterium* AS-35 or with any of the other isolated strains initially screened for their total phenols reducing capabilities when cultured in 25% and 10% (v/v) olive wastewater (sections 2.3, 2.4 and 2.5). The uninoculated control experiments showed no decrease in total phenols (data not shown).

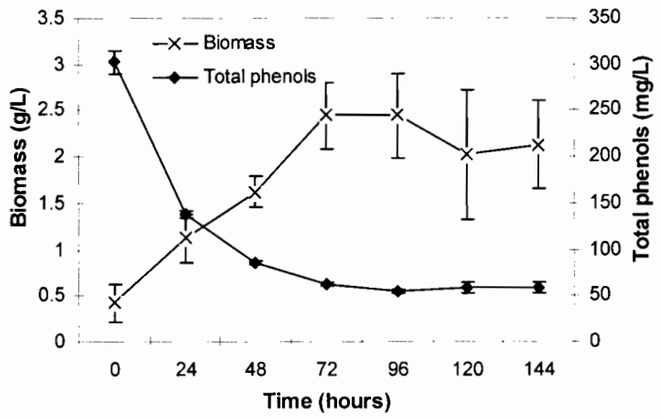



**Figure 3.26: Biomass growth and total phenols reduction of *T. pubescens* fermentations in 10% (v/v) olive wastewater.**

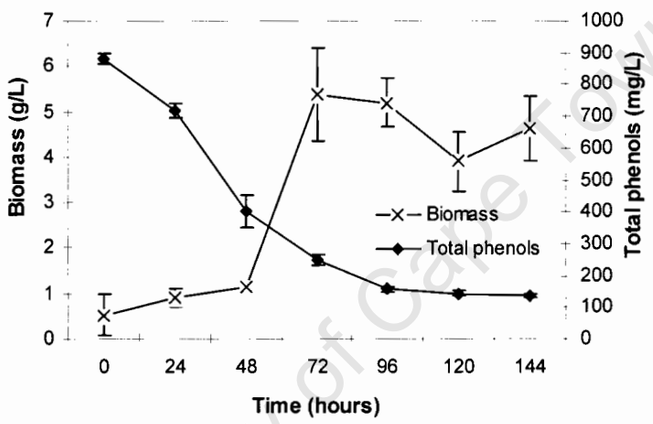



**Figure 3.27: Biomass growth and total phenols reduction of *T. pubescens* fermentations in 25% (v/v) olive wastewater.**

The COD of the wastewater was also appreciably reduced by 53 and 54% in the fermentations with 10 and 25% (v/v) olive wastewater, respectively (Table 3.4). COD reduction was more gradual in 25% (v/v) wastewater and faster in 10% (v/v) wastewater, with the majority of the COD having been reduced after 72 hours (Figs. 3.28 and 3.29).

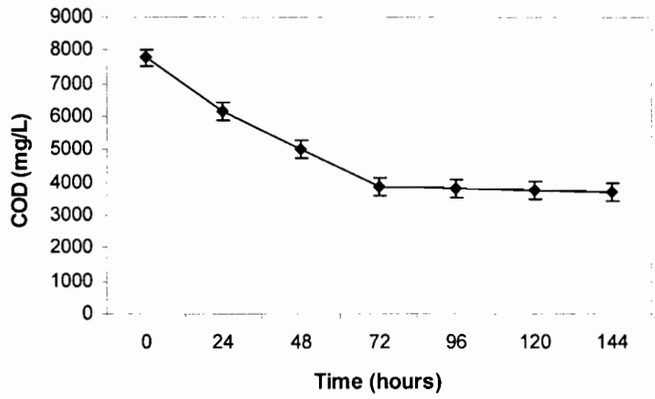



**Figure 3.28: COD reduction of *T. pubescens* fermentations in 10% (v/v) olive wastewater.**

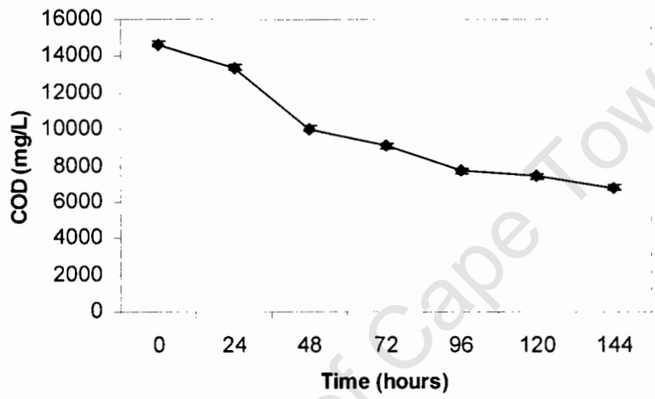



**Figure 3.29: COD reduction of *T. pubescens* fermentations in 25% (v/v) olive wastewater.**



### 3.6.3 Correlation of phenols removal and laccase production of *T. pubescens* in 10% and 25% (v/v) olive wastewater

Figures 3.30 and 3.31 show the total phenols removal versus laccase production from the fermentations with *T. pubescens* in 10% and 25% (v/v) olive wastewater. The trends observed indicated that initial phenols depletion was independent of the production of laccase. In 25% (v/v) olive wastewater, 55% of total phenols had been reduced after only 48 hours but until that point only trace amounts of laccase were detected.

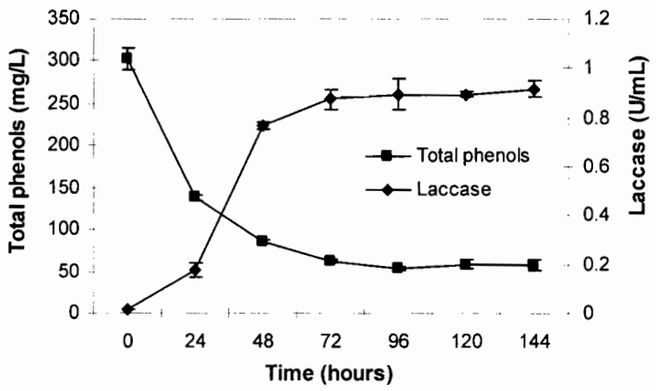



**Figure 3.30: Laccase production and total phenols reduction of *T. pubescens* fermentations in 10% (v/v) olive wastewater.**

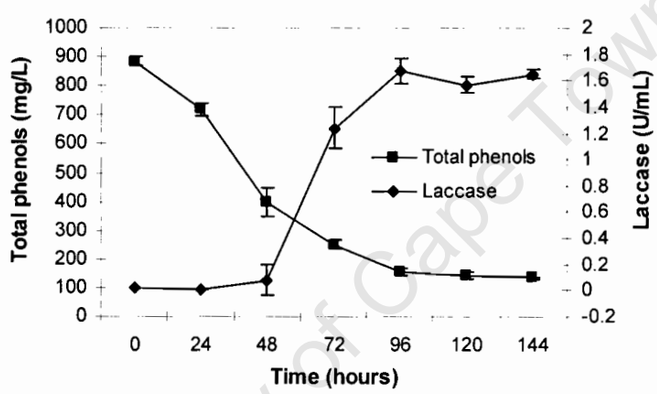



**Figure 3.31: Laccase production and total phenols reduction of *T. pubescens* fermentations in 25% (v/v) olive wastewater.**

After 48 hours, laccase production increased rapidly as the growth of the fungus entered log phase, and further phenols removal was observed, but not to the extent that was observed in the initial 48 hours of fermentation. This observation, together with the growth analyses, suggests fungal uptake and metabolism of phenols. The phenolic compounds were the major source, together with the organic acids, of utilisable carbon

and in order for the organism to produce biomass, it must metabolise the available carbon source.

In order to establish the efficiency with which *T. pubescens* removed total phenols from olive wastewater, a model, based on the Monod equation, developed by Garcia *et al.* (1997), was used. In this model the relationship between cell growth and total phenols removal is represented by the following equation:

$$X - X_0 = E_{X/F}\,(F_0 - F)$$

where $X$ and $F$ are the experimentally determined concentrations of biomass and total phenols (both in mg/L), with $X_0$ and $F_0$ being initial values. The slope of the graph of $X$ vs $F$, ($E_{X/F}$), represents the biomass produced per unit of mass of phenol compounds removed.

$E_{F/X} = 1/E_{X/F}$ is a measure of the efficiency with which the organism removes phenolic compounds.

Experimental data for the production of biomass and removal of total phenols from the investigation of the biodegradation of 10 and 25% (v/v) olive wastewater, with *T. pubescens*, were plotted (Figs. 3.32 and 3.33). The values for $E_{F/X}$ (Table 3.4) were determined to be 0.14 and 0.19 for 10% and 25% (v/v) wastewater. Although the values were higher than those obtained by Garcia *et al.* (1997), a linear relationship was not established and the $R^2$ values for these plots were very low.

The implication of this data is that the reduction in phenols observed was not only due to metabolism by the fungus. Other possible explanations for the observed reduction of phenols are the polymerisation of phenolic compounds, discussed earlier in section 3.4.1, and adsorbance of phenolic compounds to fungal mycelia resulting in the removal of measurable phenols. The colour of the fungal pellets after fermentation in olive wastewater was dark compared to the pellets from cultures of the Trametes defined

medium. The data points in figures 3.32 and 3.33 clearly show that the majority of total phenolic removal was achieved in the early stages of fermentation, with low levels of generated biomass. This suggests that the majority of phenolic compounds were removed from solution, not as a result of metabolism, but rather due to adsorption onto the biomass. The adsorption phenomenon is due to weak interactions of the mycelia and phenolic compounds and also covalent binding (Livernoche *et al.,* 1983). It is possible that the formation of covalent bonds arises from the nucleophilic attack of amino groups of the mycelial cell wall polymers (e.g., chitin) on quinones generated by the enzymatic oxidation of phenols (Wada *et al.,* 1995). Other researchers have also reported adsorption of phenolics on fungal mycelia. D'Annibale *et al.* (2004) observed progressive darkening of mycelial pellets of *P. tigrinus*, indicating chromophore adsorption, after fermentation in OMW. Aggelis *et al.* (2003) found that adsorption of phenolics, from OMW, on killed mycelia of *P. ostreatus* corresponded to approximately 8% of the initial phenols concentration. This result shows that the adsorption phenomenon does not occur only on viable biomass and plays a key role in the reduction of measurable phenols.

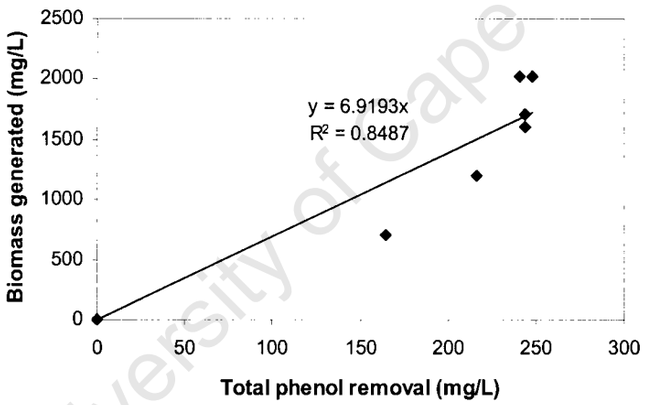



**Figure 3.32: Production of biomass and removal of total phenols over time from the fermentation of *T. pubescens* in 10% (v/v) olive wastewater.**

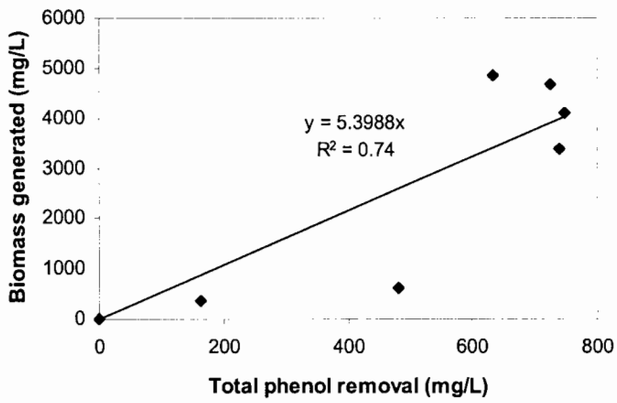



**Figure 3.33: Production of biomass and removal of total phenols over time from the fermentation of *T. pubescens* in 25% (v/v) olive wastewater.**

## 3.7 The use of laccase isolated from *T. pubescens* for the bioremediation of olive wastewater

Laccase isolated from *T. pubescens* was investigated for the reduction of phenols concentration from olive wastewater.

### 3.7.1 Production and characterisation of the laccase enzyme from *T. pubescens*

Laccase to be used in this study, was produced in 1 L Erlenmeyer flasks with 500 ml of TDM containing 2% (v/v) olive wastewater as inducer (section 2.7.1). After removing the biomass, the supernatant was freeze-dried, yielding crude enzyme. Figure 3.34 shows the SDS-PAGE analysis of the concentrated crude extract from a culture of *T. pubescens*. There is a distinct band corresponding to 65 kDa which suggested the presence of the same enzyme characterised by Galhaup *et al.* (2002). An activity gel, stained with ABTS solution, later confirmed the presence of laccase in this band (data not shown). The prominent band visible at the dye front on the gel is most likely a collection of other smaller proteins that were present in the culture broth. Laccase was not purified and the sample used for analysis was a crude extract.

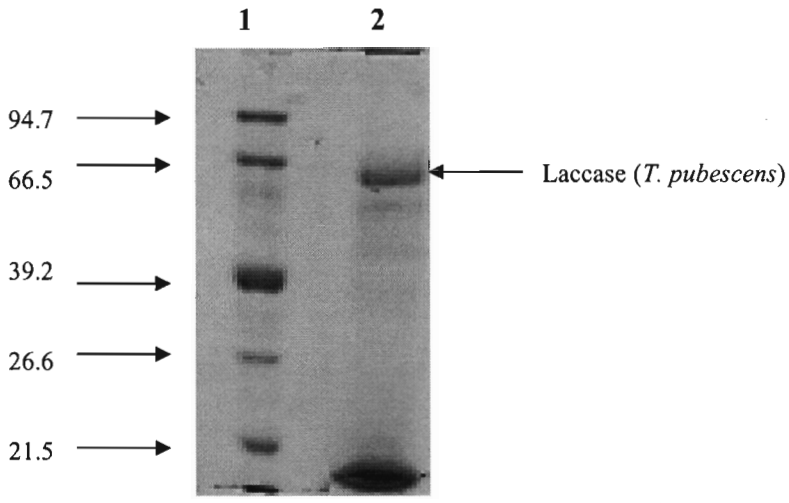



**Figure 3.34: SDS-PAGE analysis of the concentrated crude extract obtained from *T. pubescens* culture filtrate. Lane 1: molecular weight marker, lane 2: sample of crude laccase (16,1 µg protein) from *T. pubescens*.**

### 3.7.2 Investigation of the biodegradation of olive wastewater with free laccase

The advantage of an enzymatic treatment, as compared with the use of fungal cultures, is related to a shorter waste treatment period as well as the toxicity of high salt and phenols concentrations to microorganisms. The benefit that laccase has over the other ligninolytic enzymes, namely peroxidases, is that it does not require the addition of $H_2O_2$. In this investigation, crude laccase, from the supernatant of *T. pubescens* cultured in TDM with 2% (v/v) olive wastewater, was concentrated by freeze-drying the liquid and resuspending the powder in Na-acetate buffer (pH 5). This solution was dialysed against Na-acetate buffer overnight and freeze-dried to concentrate the enzyme. The dry powder was resuspended in sodium acetate buffer (pH 5). Thus, the enzyme concentration was increased 500-fold and the yield of enzyme was determined to be 67 U/g of freeze-dried crude extract. 2U and 5U of the enzyme solution were added to varying concentrations (25, 50, 75 and 100% v/v) of olive wastewater to determine the ability of the free enzyme to reduce the total phenolic fraction. Total phenols were measured before incubation with laccase and at two time intervals, namely 6 and 20 hours, after addition of enzyme. HPLC analyses were also performed on samples taken before and after addition of enzyme.

The small scale treatment of 5 ml samples of olive wastewater with concentrated, crude *T. pubescens* laccase (2U and 5U) resulted in phenols reductions of 50% at dilutions up to 75% (v/v) after only 6 hours of reaction time (Table 3.6). Phenols reductions of almost 40% were observed in undiluted olive wastewater (Table 3.6). There was no significant increase in phenols removal beyond 6 hours. However, the reduction achieved with 2U of laccase reached almost 60% in each of the dilutions of olive wastewater and as high as 64% in the 50% dilution after 20 hours (Table 3.7). The results indicate that the enzyme was not inhibited by higher initial phenols concentration.

The treatment of olive wastewater with laccase from *T. pubescens* resulted in rapid darkening of the wastewater, suggesting polymerisation. Laccase removes a single electron from the hydroxyl group of the phenolic substrates and the first oxidation products are *o*-quinones (Toscano *et al.,* 2003). These oxidation products are prone to non-enzymatic coupling reactions leading to the formation of polymers (Dec and Bollag, 1990; Toscano *et al.,* 2003).

**Table 3.6: Phenols reductions achieved after 6 hours in various dilutions of olive wastewater after the addition of 2U or 5U of laccase.**

| Concentration | % Phenolics removed | | Phenols removed (mg/L)/U | |
|---|---|---|---|---|
| | 2U | 5U | 2U | 5U |
| 25% OW | 57 | 53 | 189 | 75 |
| 50% OW | 53 | 51 | 431 | 181 |
| 75% OW | 48 | 50 | 548 | 251 |
| 100% OW | 37 | 38 | 605 | 270 |

**Table 3.7: Phenols reductions achieved after 20 hours in various dilutions of olive wastewater after the addition of 2U or 5U of laccase.**

| Concentration | % Phenolics removed | | Phenols removed (mg/L)/U | |
|---|---|---|---|---|
| | 2U | 5U | 2U | 5U |
| 25% OW | 59 | 56 | 195 | 79 |
| 50% OW | 64 | 59 | 507 | 202 |
| 75% OW | 59 | 59 | 676 | 288 |
| 100% OW | 58 | 51 | 912 | 340 |

A linear relationship was established between the initial phenols concentration and the amount of phenols removed per unit of laccase (Fig 3.35). However, the overall phenols removal was approximately the same for experiments with both 2U and 5U of laccase after 20 hours of reaction time. The reduced efficiency of phenols removal observed with the higher amount of enzyme could be as a result of the composition of the olive wastewater. Although experiments to determine the substrate specificity of the enzyme were not conducted, tyrosol and hydroxytyrosol are good substrates for laccase due to the availability of hydroxyl groups. HPLC analyses of samples taken before and after laccase treatment showed complete removal of hydroxytyrosol and tyrosol peaks (Fig. 3.36 and appendix B) and the emergence of two smaller unidentified peaks. Hydroxytyrosol and tyrosol can contribute up to 50% of the total phenols measured in olive wastewater. Therefore, after oxidation of these compounds, catalysed by laccase, and their subsequent non-enzymatic polymerisation, the total measurable phenols would have been reduced by up to 50%. This was observed in the experiments conducted and after 6 hours of reaction time the total phenols were reduced by approximately 50% in dilutions of up to 75% (v/v) (Table 3.6). Oxidation of tyrosol and hydroxytyrosol was, most likely, completed rapidly and therefore the phenols measured after 6 hours was approximately the same with 2U and 5U of enzyme. As a result, the oxidation of tyrosol and hydroxytyrosol removed the available substrates for laccase and thus, the reaction was terminated. Therefore, the reaction of laccase with olive wastewater could be substrate limited.

Another possible explanation for the reduced enzyme efficiency observed with increased amounts of enzyme could be deactivation (see Fig. 3.37). The *o*-quinones produced by laccase-catalysed oxidation of phenolic substrates might react irreversibly with some aminoacyl residues in the active site, soon after being produced, and deactivate the enzyme (Toscano *et al.*,2003). Toscano *et al.* (2003) showed that the rate of production of the *o*-quinones, from the oxidation of catechol by a phenol oxidase, is higher than the rate of their polymerisation.

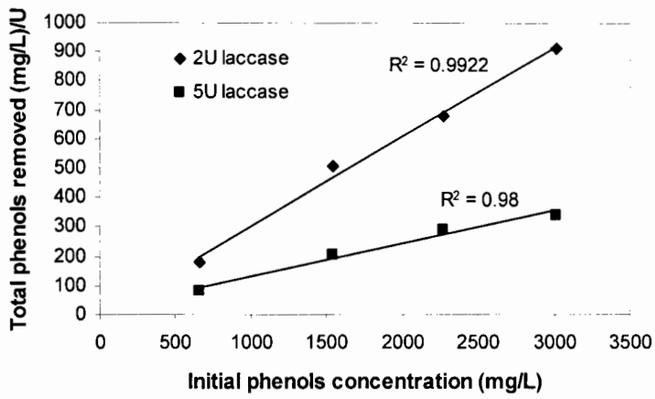
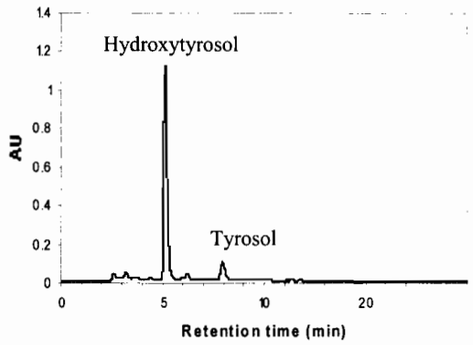



**Figure 3.35: Amount of phenols removed, after 20 hours, per unit of enzyme at varying initial phenols concentrations.**

With increased enzyme, the rate of *o*-quinone production would increase, which would result in excess oxidation products in solution allowing for an increased rate of deactivation of the enzyme. This explanation would appear to be more plausible when considering the findings of other researchers. D'Annibale *et al.* (2000) achieved increasing phenols removals from OMW with increasing amounts of laccase. Still, the data available does not show, conclusively, that the enzyme was inactivated. Further studies would need to be conducted and phenols would need to be determined at more regular intervals in order to determine the rates of reactions. Experiments would also need to be conducted to better understand the substrate specificity of laccase for the components present in olive wastewater. Previous studies have been conducted using laccase for the treatment of OMW, but to our knowledge there have been no previous investigations using laccase for the treatment of olive brine wastewaters.

**a)**




**b)**

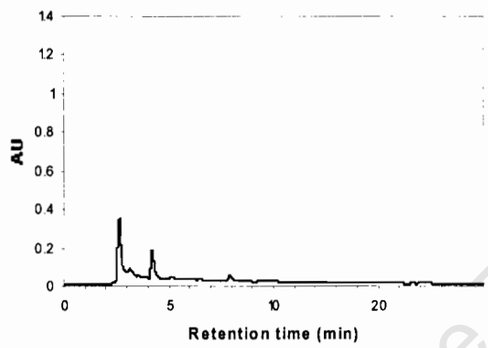



**Figure 3.36: HPLC profiles of undiluted olive wastewater before (a) and after (b) treatment with laccase (5U, 20 hours).**

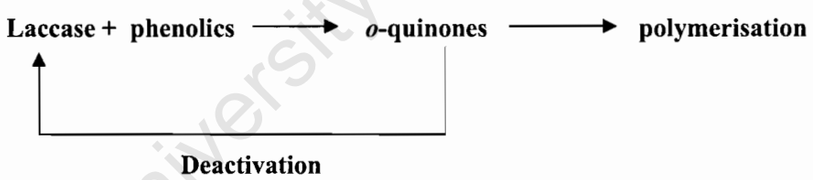



**Figure 3.37: The deactivation hypothesis reported for enzymes with phenol oxidase activity (Canovas *et al.*, 1987).**

To determine which type of treatment, either whole-cell with *T. pubescens* or enzymatic, would be the most efficient, an approximate calculation based on the data obtained in this study revealed the following:

In a 10% (v/v) dilution of olive wastewater, 1g biomass converted 0.24g phenols in 24 hours. 1g of biomass produced 369 U of laccase which was able to convert 111g phenols in undiluted olive wastewater, in a comparable amount of time. In effect, 462.5g of biomass would be required to convert the same amount (111g) of phenols. Therefore, the enzymatic treatment would appear to be significantly more efficient, in terms of phenols removal but the enzyme would still need to be produced and isolated. This would require further downstream processing, whereas the biomass, for whole-cell treatment, could be produced on a large scale and simply added to the wastewater.

# CHAPTER 4

# CONCLUSIONS

There has been little research conducted on the biodegradability of olive production wastewaters from black Kalamata olive fermentation. In fact, the majority of research conducted on the biodegradation of olive wastewaters has focused chiefly on OMW, which is the dominant olive industry in the Mediterranean region. This study is one of the few that has been conducted on the degradation of compounds present in olive fermentation wastewaters. The first main objective was to characterise the wastewater from a table olive processing plant. The olive wastewater was found to have high total phenols content, high COD, high salt content, in the order of 10 – 15% (m/v), and a low pH of approximately 4.5. The phenols present in the wastewater are known to have antimicrobial and phytotoxic effects. The olive wastewater also had low sugar and nitrogen content, with a COD: N ratio of approximately 595:1. The dark colour of the waste suggested the presence of high molecular weight, pigmented polyphenolic compounds which are recalcitrant to degradation. HPLC analyses showed that the two main simple phenolic compounds were tyrosol and hydroxytyrosol.

For the degradation studies, the first aim was to isolate strains from the evaporation ponds containing olive wastewater that are capable of degrading phenolic compounds present in the wastewater. The importance of isolating strains from the polluted site is that they are already adapted to the toxic environment of the wastewater and thus are likely to be able to metabolise the compounds present in the wastewater. Preliminary degradation studies with strains isolated from the evaporation ponds allowed us to select one strain which was efficient at degrading the compounds present in the olive wastewater. This strain (AS-35) was identified as *Bacillus megaterium*, using 16S rRNA gene techniques. It was able to reduce the total phenolic content of a 25% (v/v) dilution of the olive wastewater by 65% at a biomass concentration of 2.74 g/L. During fermentation with this strain, the medium showed a rapid increase in pH from 4.5 to 8.9, as a result of the metabolism of organic acids present in the olive wastewater. This

increase in pH was accompanied by a significant darkening of the waste, associated with the formation of larger polyphenolic compounds, a process referred to as humification. These polymerised structures are similar in size to humic acids, as indicated by size exclusion chromatography, and have a lower bioavailability than monomeric aromatic compounds. These high molecular weight compounds are understood to be responsible for the dark colour of the wastewater. Experiments were conducted with *B. megaterium* AS-35 at constant pH to determine whether the humification process could be reduced and allow for further phenols removal, but it was determined that constant pH was detrimental to the biodegradation and resulted in low phenols removals. This was an important result as it meant that a full scale treatment process would not require costly additions and therefore might prove to be a more feasible financial option.

The second aim of the degradation studies was to compare the degradation efficiencies of the locally isolated strain *B. megaterium* AS-35 with that of the commercially available white-rot fungus, *Trametes pubescens*. The comparative study was conducted using a 10% (v/v) dilution of olive wastewater in order to minimise the effect of factors such as total phenols and salinity that may inhibit growth of the organisms and to allow a realistic comparison of the growth and degradation efficiencies of the two organisms. The results showed that *T. pubescens* was more efficient at degrading the compounds present in the wastewater, compared to the bacterium, and reduced the total phenols content by up to 79% in shake flask culture compared with the 45% achieved under the same conditions by *B. megaterium* AS-35. Although size exclusion chromatography revealed that polymerization of smaller phenolic compounds into larger polyphenols occurred in fermentations with both organisms, the colour of the fermentation media from the cultures of *T. pubescens* showed a slight reduction in colour. This is thought to be a result of the degradation of compounds such as condensed tannins which would contribute to the colour of the waste.

Further studies with *T. pubescens* showed that the organism was tolerant of increased concentration of phenols and salt. The results showed that increased concentration (25% v/v) of olive wastewater enhanced growth and laccase production. The production of

laccase was related to biomass growth and maximum production was achieved during the log phase of growth in 25% (v/v) olive wastewater. Total phenols reduction and the increased biomass suggested that these two parameters were closely associated since the phenols were the most abundant carbon source present in the wastewater. Further analysis revealed that the reduction of the phenols was not solely due to their metabolism and that adsorption of phenolic compounds to the mycelia and polymerisation of phenolic compounds may also have resulted in the reduction of measurable phenols in solution. Results also indicated that initial removal of phenols from the olive wastewater was independent of extracellular laccase production since a large portion of the phenols had been removed before the onset of laccase production. Colour analyses revealed increases in absorbance at times when laccase was produced, indicating polymerisation of phenolic compounds due to the action of laccase. Up to 84% of the total phenols were reduced in 25% (v/v) olive wastewater with 0.14g (15.7%) phenols being removed per gram of biomass, which was the highest reduction obtained in any of the degradation studies conducted during the course of this project. Thus, *T. pubescens* proved to be the superior organism for the biodegradation of this waste and would be an excellent choice for an aerobic treatment of this waste.

Laccase, isolated from *T. pubescens*, was investigated for its ability to reduce the total phenolic fraction of olive wastewater. *T. pubescens* laccase (2U and 5U) resulted in phenols reductions of 50% at dilutions of 25%, 50% and 75% (v/v) and of almost 40% in undiluted olive wastewater, after only 6 hours of reaction time. After 20 hours, the phenols reduction in 25%, 75% (v/v), and undiluted olive wastewater was almost 60%, and as high as 64% in the 50% dilution. These reductions compare favourably with those achieved using advanced oxidation processes (AOPs) (Table 1.1). Benitez *et al.* (2001) reduced the phenols content of black olive brines by 20% and 52%, after 6 hours of treatment, using UV radiation and UV coupled with $H_2O_2$, respectively.

The reduction in total phenols, after treatment with laccase, can be explained by phenolic polymerisation, as indicated by the rapid darkening of the waste medium. Phenols removal was not improved with increased enzyme concentration and the reduced

efficiency with increased enzyme was suggested to be as a result of deactivation of the enzyme. The oxidation products generated by the action of laccase could have lead to irreversible reactions with the active site of the enzyme. Increased amounts of enzyme would increase the rate of production of these oxidation products and therefore increase the rate of deactivation of the enzyme. It is also proposed that the laccase reaction with the components of olive wastewater is substrate limited. For this reason, complete removal of the phenolic fraction by an enzymatic treatment with laccase is not possible.

It was observed that in order to achieve any kind of decolourisation of the wastewater the actively growing fungus had to be present. It is suggested that the fungus would metabolise products from the action of laccase and therefore remove them from solution, preventing their re-polymerisation.

The major benefit of an enzymatic treatment of olive wastewater is a significantly shorter treatment period, as well as the capacity to treat undiluted wastewater. Laccase can also be produced in an inexpensive olive wastewater medium. An approximate calculation based on the data obtained in this study revealed that 462.5g of biomass would be required to convert the same amount of phenols as achieved with laccase produced from 1g of biomass. The resulting polymer products, after enzymatic treatment, could be separated from solution and possibly incorporated into fertiliser as a source of humic matter. Conversely, whole-cell treatment requires the dilution of olive wastewater due to the antimicrobial effects of high phenols concentrations and the treatment is significantly less efficient, in terms of phenols removal, when compared to the enzymatic treatment. However, the production of the enzyme requires further downstream processing as compared to a whole-cell treatment. The biomass can be produced on large scale and added to the diluted wastewater.

Further optimisation studies on the use of laccase for the treatment of olive wastewater may provide important information for the development of an economically viable treatment process with a significantly shorter treatment time as compared to a whole cell treatment.

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## APPENDIX A

***Trametes* defined media (TDM) adapted from Addleman and Archibald (1993) contained, per litre:**

10 g glucose

5.23 g peptone

0.292 g sodium chloride

2 g potassium dihydrogen orthophosphate

0.5 g magnesium sulphate

0.1 g calcium chloride-2-hydrate

0.35 ml veratryl alcohol later replaced by 2% (v/v) olive waste

1.3 ml succinic acid dimethyl ester

10 ml Trace elements

The following trace elements were made up in 500 ml distilled water and sterilized:

0.28 g iron sulphate

0.016 g copper sulphate

0.034 g zinc chloride

0.169 g manganese sulphate

0.095 g cobalt chloride

0.0012 g nickel chloride

0.309 g ammonium molybdate

## APPENDIX B

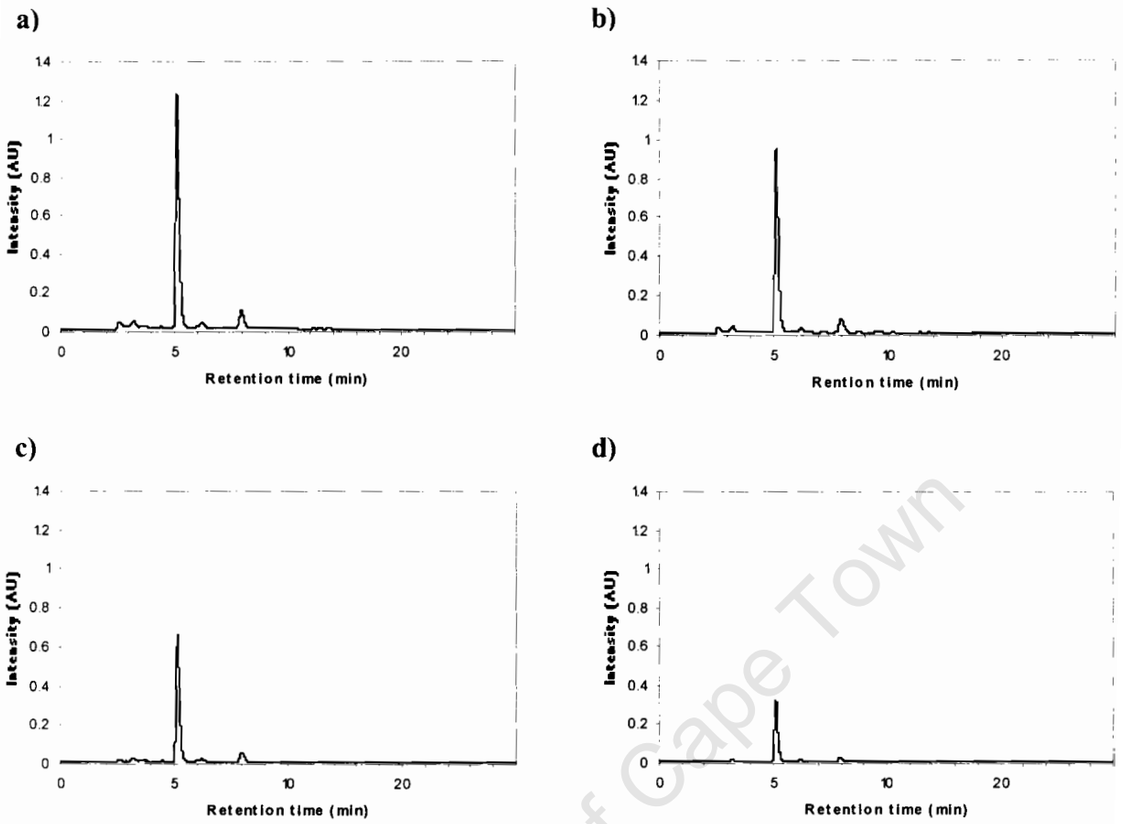


**Figure F 1. HPLC profiles of 100% (a), 75% (b), 50% (c) and 25% (d) dilutions of olive waste before incubation with laccase.**

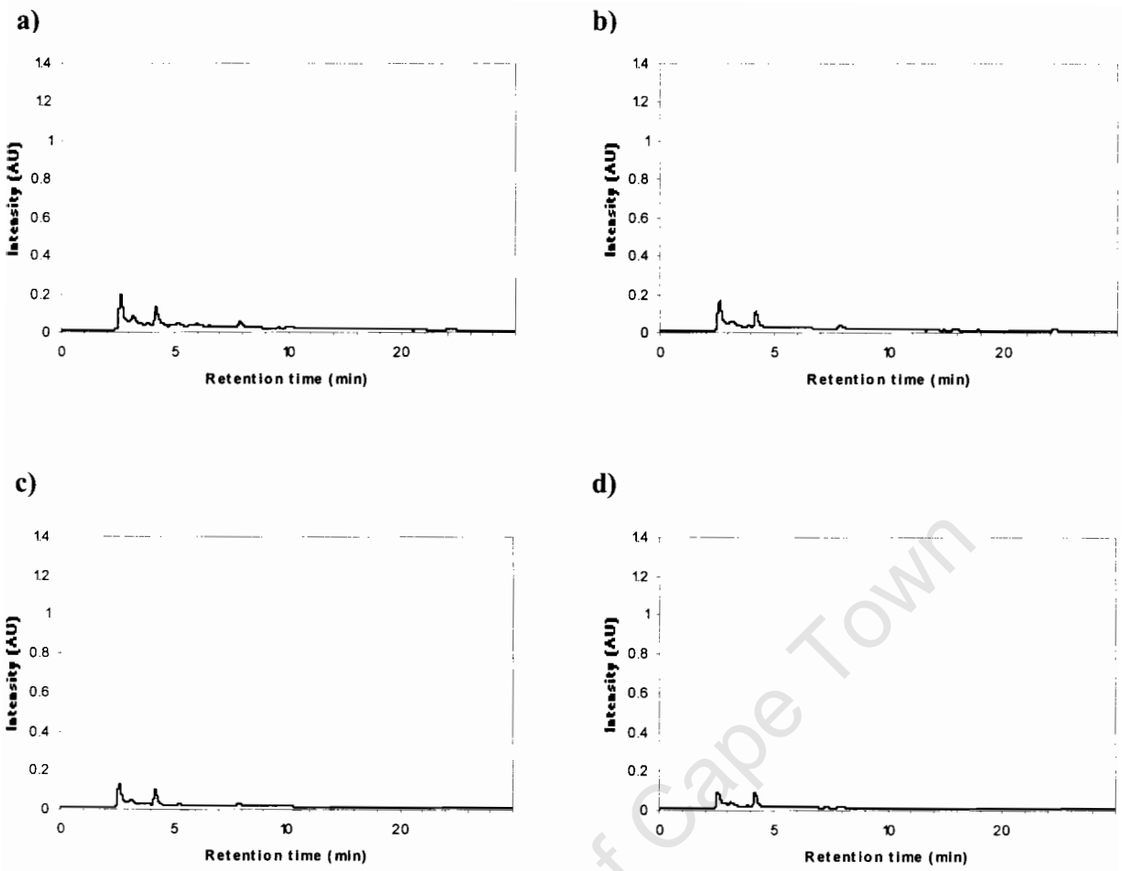


**Figure F 2. HPLC analysis of samples from 100% (a), 75% (b), 50% (c) and 25% (d) dilutions of olive waste after 20 hours of incubation with 2U of laccase.**

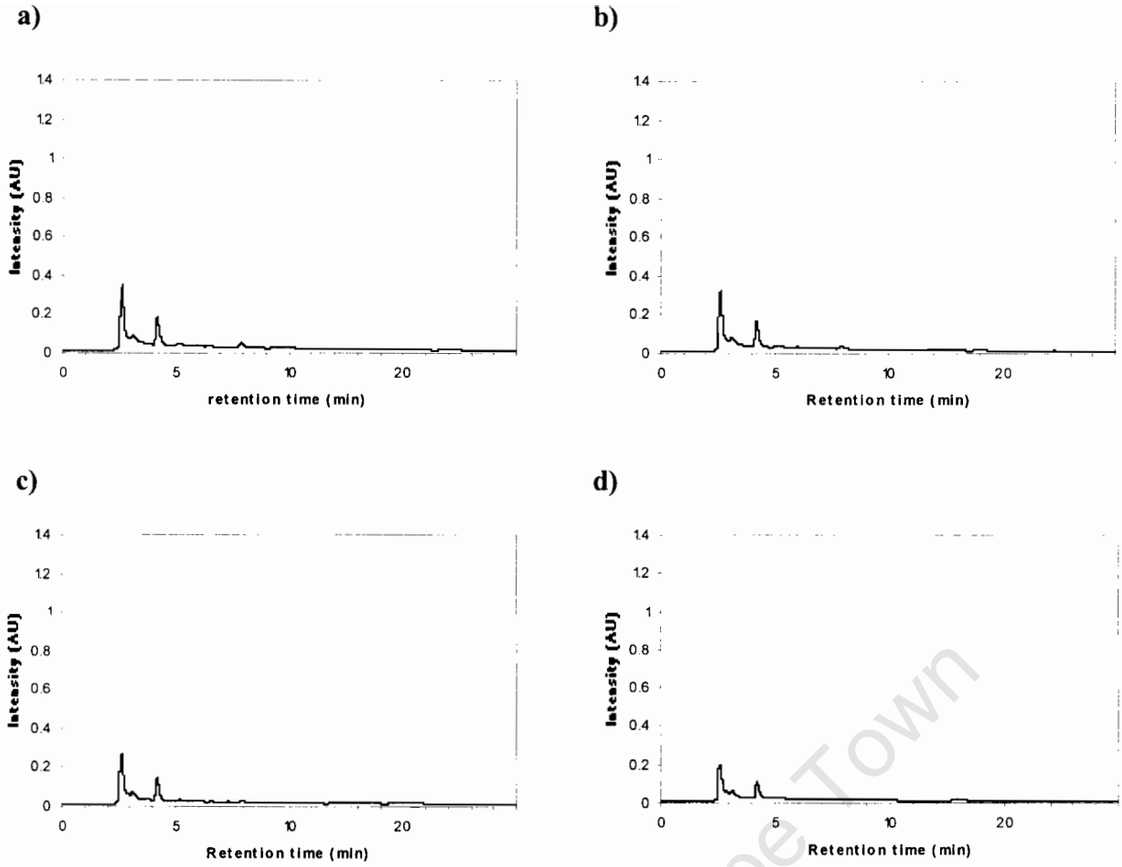


**Figure F 3: HPLC analysis of samples from 100% (a), 75% (b), 50% (c) and 25% (d) dilutions of olive waste after 20 hours of incubation with 5U of laccase.**