RHODES UNIVERSITY

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FUNGAL REMEDIATION OF DISTILLERY AND WINERY WASTEWATERS USING *TRAMETES PUBESCENS* MB 89 AND THE ENHANCED PRODUCTION OF A HIGH-VALUE ENZYME THEREIN

DEPARTMENT OF BIOCHEMISTRY, MICROBIOLOGY AND BIOTECHNOLOGY

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Abstract

In this study white-rot fungi were investigated for their efficiency at distillery wastewater remediation and the production of laccase as a valuable by-product. Distillery wastewaters are high in organic load and low in pH. The presence of phenolic compounds can lead to extremely colour-rich wastewaters and can be toxic to microorganisms. The presence of the inorganic ions may also affect biological treatment. White-rot fungi are unique among eukaryotic or prokaryotic microbes in possessing powerful oxidative enzyme systems that can degrade lignin to carbon dioxide. These ligninolytic enzymes, such as lignin peroxidase, manganese peroxidase and laccase, are capable of degrading a vast range of toxic, recalcitrant environmental pollutants and this makes the white-rot fungi strong candidates for the bioremediation of polluted soils and waters. The laccase enzyme alone has shown remediation potential in wastewaters such as beer production effluent, olive mill wastewater, alcohol distillery wastes, dye-containing wastewaters from the textile industry as well as wastewaters from the paper and pulp industry. It has been shown to be capable of remediating soils and waters polluted with chlorinated phenolic compounds, polyaromatic hydrocarbons, nitro-substituted compounds and fungicides, herbicides and insecticides.

Four white-rot fungi were initially assessed and *Trametes pubescens* MB 89 yielded better results from both a wastewater treatment and enzyme production viewpoint than *UD4*, *Ceriporiopsis subvermispora* and *Pycnoporus cinnabarinus* with the treatment of a brandy distillery wastewater and was thus selected for all further work.

Trametes pubescens MB 89 was further assessed in shake-flask cultures of 16 winery wastewaters, a wine lees, four wine-related distillery wastewaters and a distillery wastewater generated from the production of Amarula. High removal efficiencies of chemical oxygen demand, phenolic compounds and colour were obtained in both distillery and winery-related wastewaters, demonstrating the versatility of the fungal system as a pre-treatment method.

Enzymatic treatment by laccase was compared to fungal treatment. The complete fungal system displayed far superior results compared to enzymatic treatment alone High colour removal efficiencies were attained by the submerged *T. pubescens* culture, while the greatest removal by laccase was just above 10 %. The most dramatic results were obtained in the wine

lees dilutions and this serves as the best example to illustrate the differences between fungal and enzymatic treatment. The wine lees contained the highest levels of phenolic and electroactive compounds. Although laccase treatment resulted in reasonable total phenolics decreases, the colour was significantly increased, indicating that the new compounds formed by laccase were more colour-rich than the parent compounds.

Laccase was produced in high concentrations in most of the distillery wastewaters, but in low concentrations of the winery wastewaters. A bioreactor capable of treating 50 l of wastewater per batch was constructed and assessed. Laccase was produced in concentrations greater than 12000 units/l in a brandy distillery wastewater at full strength, with only an increase in pH. High concentrations of laccase were achieved using some of the distillery wastewaters. It was found that more factors than solely the presence of phenolic compounds were responsible for high laccase synthesis.

Various pH conditions, carbon sources, nitrogen sources, and cellulosic additions were assessed to determine conditions and growth medium constituents favourable for laccase synthesis by *Trametes pubescens* MB 89. A number of potential inducers and induction at two different times of induction were investigated. The investigation into enhancing laccase synthesis found that a pH modification and a number of supplementations could enhance synthesis. A different approach to inducer addition found that constant dosing until the end of the exponential growth phase enhanced laccase to a much greater extent than traditional methods of addition prior to inoculation or a few days into the active growth phase. Literature supports the notion that a high glucose concentration represses laccase synthesis. The addition of 2, 5-xylidine countered laccase suppression caused by higher glucose concentrations. However, the effectiveness of the inducers varied widely and each wastewater type would have to be assessed individually. The high variation in wastewater characteristics from batch to batch would complicate the long term utilisation of these wastewaters for commercial laccase production, even when utilising wastewater from a single source.

In conclusion, the hypothesis that the pretreatment of distillery wastewaters using laccase-producing white-rot fungi would lower the COD, colour, total phenol concentration and increase the pH can be accepted based on this study.

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Table of Contents

ABSTRACT I
ACKNOWLEDGEMENTS III
TABLE OF CONTENTS IV
LIST OF FIGURES IX
LIST OF TABLES XII
LIST OF TABLES XII

CHAPTER 1 1
INTRODUCTION AND SYNOPSIS 1
1.1 INTRODUCTION 2
1.2 SYNOPSIS 5

CHAPTER 2 11
LITERATURE REVIEW, HYPOTHESIS AND OBJECTIVES 11
2.1 LITERATURE REVIEW 12
2.1.1 Introduction to distillery and winery wastewaters 12
2.1.2 Treatment of distillery and phenolic-rich wastewaters 17
2.1.3 Physicochemical treatment processes 19
2.1.4 Extensive treatment systems 23
2.1.5 Intensive biological treatment and hybrid treatment processes 27
2.1.6 Fungal treatment of distillery and agro-industrial wastewaters 31
2.1.7 Products derived from the aerobic treatment of distillery wastewaters . 33
2.1.8 Introduction to laccase 36
2.1.9 Laccase synthesis, induction and inhibition 39
2.1.10 Utilising waste residues for laccase production 42
2.1.11 Potential industrial applications of laccase and white-rot fungi 43
2.1.12 Conclusions 44
2.2 HYPOTHESIS 47
2.3 OBJECTIVES 47

CHAPTER 3 49
SCREENING OF FOUR WHITE-ROT BASIDIOMYCETES FOR THE TREATMENT OF DISTILLERY WASTEWATER AND THE PRODUCTION OF LACCASE 49
3.1 INTRODUCTION: 50
3.2 OBJECTIVES 53

3.3 MATERIALS AND METHODS.. 53
3.3.1 CULTURES AND MAINTENANCE 53
3.3.2 SCREENING FOR BIOREMEDIATION AND LACCASE SYNTHESIS 54
3.3.3 ANAEROBIC DIGESTION OF FUNGALLY TREATED WASTEWATERS.................................. 55
3.3.4 ANALYTICAL METHODS 55
3.4 RESULTS AND DISCUSSION.. 55
3.4.1 SCREENING FOR BIOREMEDIATION AND LACCASE SYNTHESIS 55
3.4.1.1 COD removal 56
3.4.1.2 Removal of phenolic compounds.. 57
3.4.1.3 Colour removal 60
3.4.1.4 Fungal growth 62
3.4.1.5 Laccase synthesis 63
3.4.2 ANAEROBIC DIGESTION OF FUNGALLY-TREATED WASTEWATERS 66
3.5 INTERIM SUMMARY AND CONCLUSIONS... 68

CHAPTER 4 71
BIOREMEDIATION OF A BRANDY DISTILLERY WASTEWATER AND THE PRODUCTION OF LACCASE USING *TRAMETES PUBESCENS* MB 89 IN SHAKE-FLASK CULTURES AND A 50 L BUBBLE-LIFT REACTOR....................................... 71
4.1 INTRODUCTION 72
4.2 OBJECTIVES.. 73
4.3 MATERIALS AND METHODS... 73
4.3.1 CULTURE AND MAINTENANCE 73
4.3.2 FLASK CULTURES 73
4.3.3 BUBBLE-LIFT BIOREACTOR 74
4.4 RESULTS AND DISCUSSION.. 75
4.4.1 FLASK CULTURES 75
4.4.1.1 Chemical oxygen demand... 75
4.4.1.2 pH... 76
4.4.1.3 Total phenolic compounds 76
4.4.1.4 Colour.. 77
4.4.1.5 Laccase production in shake-flask cultures 78
4.4.2 BUBBLE-LIFT BIOREACTOR 79
4.4.2.1 Wastewater remediation... 79
4.4.2.2 Laccase production in the 50 l reactor 82
4.5 INTERIM SUMMARY AND CONCLUSIONS... 82

CHAPTER 5 85

FUNGAL AND ENZYMATIC TREATMENT OF FIVE DISTILLERY WASTEWATERS AND A WINE LEES 85

5.1 INTRODUCTION 86

5.2 OBJECTIVES 88

5.3 MATERIALS AND METHODS 88

5.3.1 WASTEWATER SAMPLES 88
5.3.2 FLASK CULTURES 88
5.3.3 LACCASE PURIFICATION AND TESTING 89
5.3.4 ANALYTICAL METHODS 89

5.4 RESULTS AND DISCUSSION 90

5.4.1 WASTEWATER CHARACTERISATION 90
5.4.2 PH 91
5.4.3 CHEMICAL OXYGEN DEMAND REMOVAL 92
5.4.4 CYCLIC VOLTAMMETRY AND DIFFERENTIAL PULSE VOLTAMMETRY 95
5.4.5 TOTAL PHENOLIC COMPOUNDS 95
5.4.6 COLOUR 98
5.4.7 LACCASE SYNTHESIS 100

5.5 INTERIM SUMMARY AND CONCLUSIONS 103

CHAPTER 6 105

CHARACTERISATION OF AMARULA DISTILLERY WASTEWATER, AEROBIC REMEDIATION USING *TRAMETES PUBESCENS* MB 89 AND THE SUBSEQUENT PRODUCTION OF LACCASE 105

6.1 INTRODUCTION 106

6.2 OBJECTIVES 107

6.3 MATERIALS AND METHODS 107

6.3.1 WASTEWATER CHARACTERISATION 107
6.3.2 BIOREMEDIATION AND LACCASE SYNTHESIS 108

6.4 RESULTS AND DISCUSSION 109

6.4.1 WASTEWATER CHARACTERISATION 109
6.4.2 BIOREMEDIATION 112
6.4.2.1 Chemical oxygen demand 112
6.4.2.2 Phenolic compounds 114
6.4.2.3 Colour 116
6.4.2.4 pH 118
6.4.3 LACCASE SYNTHESIS 119

6.5 INTERIM SUMMARY AND CONCLUSIONS 121

CHAPTER 7 ..... **125**

CHARACTERISATION OF SIXTEEN WINERY WASTEWATERS, ... **125**

FUNGAL REMEDIATION AND SUBSEQUENT METHANOGENIC DIGESTION **125**

7.1 INTRODUCTION **126**

7.2 OBJECTIVES **128**

7.3 MATERIALS AND METHODS **128**

7.4 RESULTS AND DISCUSSION **129**

7.4.1 WASTEWATER CHARACTERISATION 129
7.4.2 CHEMICAL OXYGEN DEMAND 132
7.4.3 CHANGE IN PH 134
7.4.4 DEGRADATION OF PHENOLIC COMPOUNDS 135
7.4.5 CHANGE IN COLOUR 137
7.4.6 LACCASE SYNTHESIS 139

7.5 INTERIM SUMMARY AND CONCLUSIONS **140**

CHAPTER 8 ..... **141**

INCREASING LACCASE SYNTHESIS USING A SUBMERGED CULTURE OF *TRAMETES PUBESCENS* MB 89 IN SYNTHETIC SOLUTION AND MODIFIED WINE-RELATED WASTEWATERS **141**

8.1 INTRODUCTION **142**

8.2 OBJECTIVES **143**

8.3 MATERIALS AND METHODS **143**

8.3.1 THE EFFECT OF PH ON LACCASE SYNTHESIS, CHEMICAL OXYGEN DEMAND REMOVAL AND TOTAL PHENOLIC COMPOUNDS REMOVAL FROM WASTEWATER 143
8.3.2 THE EFFECT OF DIFFERENT CARBON, NITROGEN, LIGNIN/CELLULOSE SOURCES AND PHOSPHORUS 144
8.3.3 THE EFFECT OF INDUCERS 144
8.3.3.1 Addition prior to or four days after inoculation *144*
8.3.3.2 Multiple additions of 2, 5-xylidine *145*
8.3.4 LACCASE SYNTHESIS IN SUPPLEMENTED DISTILLERY WASTEWATERS AND A SYNTHETIC WINE LEES 145

8.4 RESULTS AND DISCUSSION **146**

8.4.1 THE EFFECT OF PH ON LACCASE SYNTHESIS, COD REMOVAL AND TOTAL PHENOLIC COMPOUNDS REMOVAL 146
8.4.2 THE EFFECT OF DIFFERENT CARBON, NITROGEN, LIGNIN/CELLULOSE SOURCES AND PHOSPHORUS 147
8.4.3 THE EFFECT OF INDUCERS 151
8.4.3.1 Addition prior to or four days after inoculation *151*
8.4.3.2 Multiple additions of 2, 5-xylidine *153*

8.4.4 LACCASE SYNTHESIS IN SUPPLEMENTED DISTILLERY WASTEWATERS AND A SYNTHETIC WINE LEES ... 155
8.4.4.1 pH ... *156*
8.4.4.2 Copper ... *156*
8.4.4.3 Glucose ... *157*
8.4.4.4 2, 5-xylidine ... *157*
8.4.4.5 Copper, glucose and 2, 5-xylidine ... *158*
8.5 INTERIM SUMMARY AND CONCLUSIONS ... 159

CHAPTER 9 ... 163
FINAL DISCUSSION AND CONCLUSIONS ... 163
9.1 OVERALL DISCUSSION ... 164
9.2 WASTEWATER TREATMENT ... 166
9.3 LACCASE SYNTHESIS ... 172
9.3.1 NONSUPPLEMENTED WASTEWATERS ... 172
9.3.2 ENHANCING LACCASE SYNTHESIS ... 175
9.3.3 WASTEWATER SUPPLEMENTATION ... 177
9.4 CONCLUSIONS ... 179
9.5 RECOMMENDATIONS FOR FURTHER WORK ... 182

CHAPTER 10 ... 185
REFERENCES ... 185

APPENDIX A.1 ... 205
APPENDIX A.2 ... 206
APPENDIX B ... 207
APPENDIX C ... 211

List of Figures

Figure 3.1: Change in COD for the raw (□) and PVPP-treated (Δ) wastewaters inoculated with a) *Trametes pubescens*, b) *Ceriporiopsis subvermispora*, c) *Pycnoporus cinnabarinus* and d) *UD4* (results are from the pooling of three separate samples). 57
Figure 3.2: Change in total phenols for the liquid culture inocula of *Trametes pubescens, Ceriporiopsis subvermispora*, *Pycnoporus cinnabarinus* and *UD4* and in raw (Δ) and PVPP-treated (□) wastewater and in the control (◊). 58
Figure 3.3: Raw WW, b: Raw WW after centrifugation and filtration, c: Raw WW after centrifugation, filtration, pH adjustment to 5.3 and autoclaving, d: PVPP-treated WW, e: PVPP-treated WW after pH adjustment to 5.3 and autoclaving and f: distilled water (control). 61
Figure 3.4: Wastewater colour before and after PVPP and fungal treatment. 1: Raw wastewater, 2: Raw WW after fungal treatment, 3: PVPP-treated wastewater, 4: PVPP-treated WW after fungal treatment, 5: distilled water and 6: controls in distilled water. 61
Figure 3.5: Laccase activities for the liquid culture inocula of *Trametes pubescens* in PVPP-treated wastewater (PVPP) (*n* = 4) and raw wastewater (Raw) (*n* = 4) from biomass that was exposed to a 10 % concentration of wastewater and no prior wastewater exposure. 66
Figure 3.6: Change in pH for the anaerobic digestion of the fungally-treated wastewater (*n* = 4), fungally and PVPP-treated wastewater (*n* = 4) . Raw wastewater (*n* = 1) and PVPP-treated wastewater (*n* = 1) that were not treated by fungi served as controls. 68

Figure 4.1: Schematic diagram of 50 l bubble-lift bioreactor. 75
Figure 4.2: Change in pH and COD of wastewater plotted with dry mass during shake-flask treatment of brandy distillery wastewater using *T. pubescens*. Error bars indicate standard deviation and in some cases are too small to be visible. 76
Figure 4.3: Change in total phenolic compounds concentration (left) and colour (right) over a 15 day period. 77
Figure 4.4: Production of laccase (◆) (*n* = 4) and dry mass (□) (*n* = 1) by *T. pubescens* in flask cultures. Error bars for laccase production indicate standard deviation. 78
Figure 4.5: Change in a) COD and b) pH of wastewater during a 15.5 day period of fungal treatment. Std dev <5 % (duplicate samples taken at same time). Vertical dotted lines indicate interruptions in aeration. 80
Figure 4.6: Change in a) total phenolic compounds and b) colour of wastewater during a 15.5 day period of fungal treatment. Std dev <5 % (duplicate samples taken at same time). Vertical dotted lines indicate interruptions in aeration. 801
Figure 4.7: Production of laccase (◆) (*n* = 4) and dry mass (□) (*n* = 1) by *T. pubescens* in the 50 l bubble-lift reactor. Error bars indicate standard deviation. 82

Figure 5.1: Wastewater COD values before and after fungal treatment for wastewaters at percentage concentration. Error bars represent standard deviation and in some cases are too small to be visible. 92
Figure 5.2: Laccase synthesis for all wastewaters and concentrations in which *T. pubescens* synthesised laccase at concentrations greater than 1800 units/L....................102

Figure 6.1: Change in COD (mg/L) over a 14 day period for various Amarula wastewater concentrations inoculated with *T. pubescens.* Highest standard deviation: 0.86 g/L....................112
Figure 6.2: Total mass COD removed (mg/L) by day four for various Amarula wastewater concentrations inoculated with *T. pubescens*. Error bars represent standard deviation. 113
Figure 6.3: Change in total phenolic compounds concentration over a 14 day period for various Amarula wastewater concentrations inoculated with *Trametes pubescens.* Highest standard deviation: 11 mg/L 114
Figure 6.4: Change in colour over a 14 day period for various Amarula wastewater concentrations inoculated with *Trametes pubescens*. Highest relative standard deviation: 14 %....................117
Figure 6.5: Change in pH over a 14 day period for various Amarula wastewater concentrations inoculated with *Trametes pubescens*. Highest standard deviation: 0.21 pH units. 118
Figure 6.6: Laccase activity over a 14 day period for various Amarula wastewater concentrations inoculated with *Trametes pubescens*. Highest standard deviation: 130 units/L. 120

Figure 7.1: Change in COD after aerobic fungal digestion (after 7 or 9 days for E, G, J, K and M) of undiluted winery wastewaters. Error bars represent standard deviation.. 132
Figure 7.2: Decrease in COD with methanogenic digestion of fungally-treated and raw wastewater after 14 days.. 133
Figure 7.3: Change in total phenolic compounds concentration after aerobic fungal digestion (final concentration after 7 or 9 days for E, G, J, K and M) of undiluted winery wastewaters. Error bars represent standard deviation....................136
Figure 7.4: Change in concentrations of total phenolic compounds with methanogenic digestion of fungally-treated and raw wastewater.. 137
Figure 7.5: Change in colour after aerobic fungal digestion (after 7 or 9 days for E, G, J, K and M) of undiluted winery wastewaters. Error bars represent standard deviation.. 137
Figure 7.6: Change in colour with methanogenic digestion of fungally-treated and raw wastewaters after 14 days....................138
Figure 7.7: Highest laccase activities (after 7 or 9 days for E, G, J, K and M) resulting from the treatment of undiluted winery wastewaters. Error bars represent standard deviation and *indicates samples that increased in laccase activity.. 139

Figure 8.1: Total phenols and COD removal efficiencies (%) after twelve days and relative percentage of highest laccase activities (normalised against the laccase maximum at pH 5.0 of 6391 ± 332 units/l). Error bars indicate standard deviation. 146
Figure 8.2: Laccase synthesis of *Trametes pubescens* in various supplemented wastewaters. Error bars indicate standard deviation. Raw: unmodified wastewater, pH 5: raw wastewater at pH 5, Cu: additional copper at 1 mM, Gl: additional glucose at 20 g/l, 2, 5-X: additional 2, 5-xylidines added at 1 mM per day for three days and 2, 5-X, Gl+Cu: combination of glucose, copper and 2, 5-xylidine. 155

Figure B.1: Results from CV (CV vs. Ag/AgCl) of wastewater B1 showing a small peak at 0.4V. 207
Figure B.2: Results from CV (CV vs. Ag/AgCl) of wastewater B2 showing a small peak at 0.4V. 207
Figure B.3: Results from CV (CV vs. Ag/AgCl) of wastewater S1 showing two peaks, indicating two electro-active compound present in this sample. 207
Figure B.4: Results from CV (CV vs. Ag/AgCl) of wastewater S2 showing a small peak at 0.395V. 208
Figure B.5: Results from CV (CV vs. Ag/AgCl) of wastewater DL. The lack of any distinguishable peaks indicates no electro-active compounds were present. 208
Figure B.6: Results from CV (CV vs. Ag/AgCl) of wastewater L showing a major peak at about 0.4V and high concentrations of electro-active compounds at higher potentials. 208
Figure B.7: Results from DPPV (DPPV vs. Ag/AgCl) of wastewater B1 showing small peaks at 0.38V and at 0.8V indicating low concentrations of electro-active compounds. 209
Figure B.8: Results from DPPV (DPPV vs. Ag/AgCl) of wastewater B2 showing peaks at 0.38V and one at 0.68V, but at very low concentrations. 209
Figure B.9: Results from DPPV (DPPV vs. Ag/AgCl) of wastewater S1 showing a highly electro-active compound at 0.37V. Another peak at 0.8V was only detectable at higher concentrations. 209
Figure B.10: Results from DPPV (DPPV vs. Ag/AgCl) of wastewater S2 showing a small peak at 0.38V. More than one electro-active compound was present (due to the width and the bump to the right of peak). 210
Figure B.11: Results from DPPV (DPPV vs. Ag/AgCl) of wastewater DL showing an electro-active compound 0.8V, but it is only evident at higher concentrations. 210
Figure B.12: Results from DPPV (DPPV vs. Ag/AgCl) of wastewater L showing a very broad peak at 0.38V. This sample contained highly electro-active compounds. 210

List of Tables

TABLE 2.1: POTENTIAL ENVIRONMENTAL IMPACT OF WINERY AND/OR DISTILLERY WASTES (ADAPTED FROM EPA, 2004)....14

TABLE 2.2: TABLE OF THE MAJOR FLAVONOID AND NON-FLAVONOID PHENOLIC COMPOUNDS FOUND IN WINES. 16

TABLE 3.1: CHEMICAL OXYGEN DEMAND REMOVAL EFFICIENCIES FOR THE FOUR FUNGI IN RAW AND PVPP-TREATED WASTEWATER (RESULTS ARE FROM THE POOLING OF THREE SEPARATE SAMPLES)....56

TABLE 3.2: HIGHEST TOTAL REMOVAL EFFICIENCIES OF PHENOLIC COMPOUNDS OBTAINED IN RAW AND PVPP-TREATED WASTEWATER. VALUES REPRESENT MEANS ± STANDARD DEVIATION ($N = 3$)....58

TABLE 3.3: COLOUR REMOVAL EFFICIENCIES (%) OBTAINED IN RAW AND PVPP-TREATED WASTEWATER (RESULTS ARE FROM THE POOLING OF THREE SEPARATE SAMPLES). 60

TABLE 3.4: COMPARISON OF MASS AND MASS INCREASE OBTAINED OF THE FOUR FUNGI. VALUES REPRESENT MEANS ± STANDARD DEVIATION ($N = 3$)....63

TABLE 3.5: HIGHEST LACCASE ACTIVITIES (UNITS/L) PRODUCED IN RAW AND PVPP-TREATED WASTEWATER. VALUES REPRESENT MEANS ± STANDARD DEVIATION ($N = 3$). 64

TABLE 3.6: COMPARISON OF RESULTS OF COD REMOVAL OBTAINED WITH FUNGAL TREATMENT, ANAEROBIC DIGESTION AND A COMBINATION OF BOTH METHODS FOR TREATMENT OF PVPP-PRETREATED AND RAW WASTEWATER (INITIAL COD VALUE: 25.5 G/L COD AFTER PVPP TREATMENT: 22.5 G/L). 67

TABLE 5.1: RESULTS FOR WASTEWATER CHARACTERISATION TESTS....91

TABLE 5.2: RESULTS FROM DIFFERENTIAL PULSE VOLTAMMETRY AND CYCLIC VOLTAMMETRY IN RELATION TO TOTAL PHENOLS. 93

TABLE 5.3: ORIGINAL TOTAL PHENOLIC COMPOUNDS CONCENTRATIONS AND PERCENTAGE ENZYMATIC AND FUNGAL REMOVAL. 96

TABLE 5.4: CHANGE IN WASTEWATER COLOUR AFTER ENZYMATIC AND FUNGAL TREATMENT. 99

TABLE 5.5: MAXIMUM LACCASE ACTIVITY, DAYS ON WHICH MAXIMA OCCURRED AND RELATIVE FOLD INCREASE. 101

TABLE 6.1: CHARACTERISATION OF THE WASTEWATER (MG/L UNLESS STATED OTHERWISE). 109

TABLE 7.1: CHARACTERISTICS OF WINERY WASTEWATERS. TOTAL PHENOLS, COD, NH_3, NO_2^-, NO_3^- AND CL^- (MG/L) AND COLOUR (HAZEN). A SINGLE 25 L SAMPLE WAS COLLECTED AND THIS WAS TESTED IN TRIPLICATE....131

TABLE 7.2: PH VALUES OF WASTEWATERS BEFORE AND AFTER FUNGAL DIGESTION (AFTER 7 OR 9 DAYS FOR E, G, J, K AND M) AND METHANOGENIC DIGESTION (AFTER 14 DAYS).. 135

TABLE 8.1: RESULTS OBTAINED FROM CARBON, NITROGEN, LIGNIN/CELLULOSE SOURCES AND PHOSPHORUS. 148

TABLE 8.2: COMPONENTS OF THE SYNTHETIC GROWTH MEDIUM....150

TABLE 8.3: RESULTS OBTAINED BY THE ADDITION OF INDUCERS AT INOCULATION OR FIVE DAYS THEREAFTER. 151
TABLE 8.4: RESULTS OBTAINED WITH VARYING 2, 5-XYLIDINE ADDITION. 153
TABLE 8.5: AVERAGED DATA OF THE RESULTS OBTAINED WITH VARYING INDUCER ADDITION TO THE FOUR WASTEWATERS. 155

TABLE 9.1: ACCEPTABLE DISCHARGE LIMITS ACCORDING TO STELLENBOSCH MUNICIPAL BYLAWS................................165
TABLE 9.2: WASTEWATER TREATMENT AND LACCASE PRODUCTION BY *TRAMETES PUBESCENS* MB 89 IN DIFFERENT WASTEWATERS. 166
TABLE 9.3: LACCASE PRODUCTION BY *TRAMETES PUBESCENS* MB 89 IN DIFFERENT WASTEWATERS. 173

TABLE A.1: RESULTS FOR TOTAL PHENOLIC COMPOUNDS CONCENTRATION IN WATER, 20 % WINE CONCENTRATION AND 20 % WINE CONCENTRATION WITH 750 MG/L PURE PHENOL...205
TABLE A.2: RESULTS OF PHENOLS ADSORBED AND DESORBED (NG/100MG RESIN). 206

Glossary

Activated carbon

This is a general term of a carbon material derived mainly from charcoal. It has an exceptionally high surface area and is used in gas purification, gold purification, metal extraction, water purification, medicine, sewage treatment for air filtration.

ANZECC

The Australian and New Zealand Environment Conservation Council was a Ministerial Council that operated between 1991 and 2001. ANZECC provided a forum for member governments to develop coordinated policies about national and international environment and conservation issues.

ARMCANZ

Agriculture and Resource Management Council of Australia and New Zealand

Cyclic voltammetry

CV is a type of potentiodynamic electrochemical measurement where a voltage is applied to a working electrode in solution and current flowing at the working electrode is plotted versus the applied voltage to give the cyclic voltammogram. Cyclic voltammetry can be used to study the electrochemical properties of species in solution as well as at the electrode/electrolyte interface. In a cyclic voltammetry experiment a potential is applied to the system, and the faradaic current response is measured (a faradaic current is the current due to a redox reaction). The current response over a range of potentials (a potential window) is measured, starting at an initial value and varying the potential in a linear manner up to a pre-defined limiting value. At this potential (often referred to as a switching potential), the direction of the potential scan is reversed, and the same potential window is scanned in the opposite direction (hence the term cyclic). This means that, for example, species formed by oxidation on the first (forward) scan can be reduced on the second (reverse) scan. This technique is commonly used, since it provides a fast and simple method for initial characterization

of a redox-active system. In addition to providing an estimate of the redox potential, it can also provide information about the rate of electron transfer between the electrode and the analyte, and the stability of the analyte in the electrolyzed oxidation states (e.g., whether or not they undergo any chemical reactions).

Differential pulse voltammetry

With DPV the current is measured immediately before each potential change and the current difference is plotted as a function of potential. These sampling points are selected to allow for the decay of the nonfaradaic (charging) current. The difference between current measurements at these points for each pulse is determined and plotted against the base potential. Differential pulse voltammetry has found excellent usage in the identification of electrolysed species and is more sensitive than cyclic voltammetry.

DWAF

The Department of Water Affairs and Forestry is the custodian of South Africa's water and forestry resources.

Glacé

Glacé fruit is a term for fruit preserved in sugar syrup. A *marron glacé* is a confection consisting of a chestnut candied in sugar syrup and glazed.

Heterologous

Derived from a different origin or relating to cytologic or histological elements that do not normally occur in a designated part of the body (i.e. consisting of dissimilar tissue, as that of another species or that of a tumor).

Inducer

Many different molecules can effect the level of gene expression by promoting or preventing transcription. Repressor proteins bind to the DNA strand and prevent DNA polymerase from being able to attach to the DNA and synthesize mRNA. Inducers

bind to repressors, causing them to change shape and preventing them from binding to DNA. Therefore, they allow transcription, and thus gene expression, to take place. Some inducers are modulated by activators, which have the opposite effect on gene expression as repressors. Inducers bind to activator proteins, allowing them to bind to the DNA strand where they promote RNA transcription.

Lees

Deposits of dead yeast or residual yeast and other precipitates to the bottom of a vat of wine after fermentation and aging.

Madereization

Is a term used to describe flavour alterations and colour intensification that occurs in red wines and red wine musts due to oxidative reactions involving polyphenols, aldehydes, amino acids and proteins, which are stimulated by iron, copper and enzymes.

Mediator

A compound with a low molecular weight that can be oxidized by laccase to form a stable radical, which in turn may oxidize other compounds that in principle are not substrates of laccase.

Pressmud

A solid residue byproduct obtained from sugarcane juice before crystallization of sugar and is generally used as fertilizer or for extraction of wax.

Rooibos

Aspalathus linearis is a broom-like member of the legume family of plants and is used to make a herbal tea.

Notation

^{14}C	Carbon 14
ABTS	2,2'-azino-di-[3-ethylbenzthiazoline sulphonate (6)]
AGDEWR	Australian Government Department of the Environment and Water Resources
BOD	Biological (biochemical) oxygen demand
COD	Chemical oxygen demand
CTAB	Cetyltrimethylammonium bromide
DWAF	Department of Water Affairs
EPA	Environment Protection Authority
GWhe/a	Gigawatt-hours electrical energy annually
H_2O_2	Hydrogen peroxide
HRT	Hydraulic retention time
Na_2CO_3	Sodium carbonate
OMW	Olive mill wastewaters
PHB	Poly *b*-hydroxybutyric acid
PVPP	Polyvinylpolypyrrolidone
RBC	Rotating biological contactor
TIFP	Technical information of fruit pulp
TOC	Total organic carbon
TSS	Total suspended solids
VFA	Volatile fatty acid

Chapter 1

Introduction and synopsis

1.1 Introduction

A great volume of wastewater is produced globally by the distillery and the winery industries. Wastewaters are generated from distillation in order to produce ethanol or engender a higher ethanol concentration in liquor. Approximately eight to fifteen litres of wastewater are generated by molasses-based distilleries for every litre of ethanol produced (Nataraj *et al*., 2006). The agricultural products utilised to produce alcoholic beverages and purified ethanol include maize, sugarcane, potatoes, wheat, barley and grapes. The amount of aqueous waste produced by the distillery industry is increasing greatly due to growing, and in some cases enforced demand for ethanol, such as for use in biofuels.

Environmental pollution from wastewater release is a threat to public and environmental health of which the public is now acutely aware, due to media coverage and the modern ease of information transfer. Punitive measures and public perception of environmental impact have catalysed a major industry for environmental remediation of currently produced wastes as well as sites that have been polluted over many years. Biological degradation has become increasingly popular as it is seen as benign and as having a lower environmental impact than physical and chemical alternatives. Bioremediation methods utilising microorganisms and/or their enzymes are replacing traditional systems such as the chemical or physical methods historically used in waste and wastewater treatment processes.

White-rot fungi have been shown to exhibit unique biodegradation capabilities. This is primarily due to their production of extracellular, broad substrate range enzymes that are capable of degrading lignin, a recalcitrant biopolymer. Lignin peroxidase, manganese peroxidase and laccase are the three key enzymes involved in lignin degradation. These enzymes enable the fungi to tolerate and degrade many recalcitrant compounds that are normally toxic or inhibitory to other microorganisms. With the aid of these three enzymes and smaller mediatory molecules, white-rot fungi are capable of oxidising lignin to carbon dioxide.

One of the key fungal enzymes in lignin degradation is laccase. Laccase is a dimeric or tetrameric glycoprotein belonging to a group of multi-nuclear copper-containing blue

oxidases, usually containing four copper atoms per monomer (Yarapolov *et al*., 1994). Laccase catalyses the oxidation of various organic compounds with the reduction of molecular oxygen to water. It does this without producing or requiring hydrogen peroxide. The substrate radicals formed are highly reactive and can polymerise with one another and eventually precipitate out of solution due to polymer hydrophobicity. Laccase has a broad substrate range including *ortho* and *para*-diphenols and aromatic amines (Thurston, 1994). It has high catalytic constants and its use of molecular oxygen as a second substrate gives it a broad scope of applicability in bioremediation, organic synthesis, immunoassays and biosensors (Saito *et al*., 2003). This wide range of applications of laccase is due to its broad substrate range, that it requires only molecular oxygen as an electron donor and does not produce or require peroxides during the reaction.

Laccase was originally discovered in the exudates of the Japanese lacquer tree, *Rhus vernicifera* (Yoshida, 1883) and was subsequently identified in plant, fungal, bacterial and insect sources (Thurston, 1994). By 2006 more than 100 had been identified from fungal sources alone (Baldrian, 2006). Fungal specimens are known to secrete high concentrations of enzymes. Ascomycete, deuteromycete and basidiomycete divisions are all known to contain laccase secretors. The basidiomycetes are known to contain some of the most potent laccase producers (Bollag and Leonowicz, 1984) and inhabit a wide variety of niches ranging from woodlands to marine environments. They grow on wood as well as in soil and the class contains genera such as *Pycnoporus*, *Trametes*, *Ganoderma* and *Pleurotus*. The extracellular secretion of the enzyme is advantageous to a commercial production process, as it reduces the complexity of purification by limiting the need for an extraction step. Additionally, with certain fungal cultures the secretion of a single enzyme is beneficial as it simplifies downstream processing by reducing the need for protein separation.

Laccase is an industrially important enzyme with numerous potential applications, both for the enzyme itself and for systems incorporating the fungus that produces the enzyme. Laccase has shown remediation potential for beer production wastewaters (Yague *et al*., 2000), olive mill wastes (Jaouani *et al*., 2005; Martirani *et al*., 1996), alcohol distillery wastewaters (Fitzgibbon *et al*., 1998), dye-containing wastewaters from the textile industry (Blanquez *et*

al., 2004; Hou *et al*., 2004; Wesenberg *et al*., 2003; Robinson *et al*., 2001; Pointing and Vrijmoed, 2000), in lignopolystyrene graft polymer degradation (Milstein *et al*., 1992), and with pulp bleaching and delignification and Kraft pulp wastewater treatment in the paper and pulp industry (Archibald *et al*., 1997). Laccase may also be used to stabilise wines (Minussi *et al*., 2007), beers (Mathiasen, 1995) and fruit juices (Cantarelli and Giovanelli, 1990).

Cheaper production of the enzyme may further increase its use, by making it affordable to industrial applications that are currently hampered by the high costs associated with the purified enzyme. Additionally, if a waste residue is used to produce the enzyme the by-product value can offset the treatment process costs. One such example is fungal treatment of distillery wastewater, which has the potential to decrease the concentrations of potentially inhibitory phenolic compounds, colour and chemical oxygen demand (COD) and raise the pH of the wastewater. Concomitant laccase synthesis can be stimulated by the presence of the phenolic compounds, thereby producing a valuable by-product from the treatment of a wastewater. Using the wastewater as a growth medium for the production of enzymes has a dual purpose: the wastewater, known for its harmful environmental characteristics, is remediated by the fungal metabolism and enzymes are simultaneously produced. Distillery wastes have a high concentration of phenolic compounds, a high COD, are rich in colour, acidic and vary widely according to the raw material distilled. These characteristics make them difficult to treat by conventional anaerobic digestion. Pretreating distillery wastewater by aerobic digestion with laccase-secreting fungi decreases the polyphenol, COD and colour content and raises the pH. This renders the wastewater less toxic to secondary treatment by anaerobic digestion. The polyphenols, which normally inhibit bioremediation, can induce high-value enzyme production when the correct microorganisms are used.

The distillery wastewaters originating as a result of ethanol removal offer a unique potential growth substrate for the production of lignolytic enzymes such as fungal laccase. The wastewater can be considered as a medium containing various components capable of supporting fungal growth, while simultaneously possessing an array of lignin-related compounds that are known inducers of laccase in white-rot fungi. In addition to containing compounds that may be utilised for growth and enzyme production, the distillation process

produces a heat-sterilised medium, thereby decreasing the costs associated with media preparation.

1.2 Synopsis

This study dealt with the fungal treatment of a variety of wastewaters originating from the production of brandy and Amarula, ethanol distillation from low-grade wine and wine lees distillation, and sixteen wastewaters originating from wine production. Wastewaters were remediated by *Trametes pubescens* MB 89 as well as a partially purified laccase from *Trametes pubescens* MB 89. Some of the fungally-treated wastewaters were also assessed by post-treatment with methanogenic digestion to determine whether pre-treatment had rendered the wastewater less recalcitrant and inhibitory to anaerobic digestion.

The literature pertaining to winery and distillery wastewater characteristics and treatment methods are reviewed in Chapter 2. These wastewaters are generally acidic, high in COD and colour, and may contain phenolic compounds that can inhibit biological treatment systems. Treatment of distillery and phenolic compound rich wastewaters by physicochemical, extensive and intensive aerobic biological systems, hybrid treatment methods as well as products derived from intensive fungal treatment are also discussed. White-rot fungi have been shown to exhibit unique biodegradation capabilities, primarily due to their production of extracellular and broad-substrate range enzymes that are capable of mineralising lignin, a recalcitrant biopolymer. One of these enzymes, laccase, catalyses the oxidation of various organic compounds with the subsequent reduction of molecular oxygen to water. Laccase synthesis, induction and inhibition are discussed with the utilisation of waste residues for laccase production and the enzyme's potential industrial applications. Distillery wastewaters offer a unique, pre-sterilised, potential growth substrate for the production of lignolytic enzymes such as laccase. Compounds may be utilised for enzyme and biomass production, thereby resulting in remediation by the growing fungus.

Chapter 3 examines the potential of submerged cultures of four white-rot fungi for the treatment of a brandy distillery wastewater and assesses whether the compounds present in the wastewater would allow for laccase synthesis. *Trametes pubescens* MB 89, *Ceriporiopsis*

subvermispora, *Pycnoporus cinnabarinus* and *UD4* were screened for their ability for the bioremediation of a raw distillery wastewater as well as distillery wastewater that had been pre-treated by polyvinylpolypyrrolidone (PVPP). The PVPP-pre-treatment removed a substantial portion of the phenolic compounds, which have been implicated in other wastewaters as the cause for inhibition of biological remediation systems. The potential of each strain was measured as a function of its capacity for decreasing the COD, total phenolic compounds concentration and the colour of the wastewater, while simultaneously producing laccase in high concentrations. *Trametes pubescens* MB 89 outperformed the other three white-rot fungi in both remediation and laccase production. The PVPP pre-treatment negatively affected laccase synthesis and the only benefit displayed was a marginal increase in colour removal.

In Chapter 4 *T. pubescens* MB 89 was used further in flask cultures and attained 79 ± 1.1 % COD removal, 80 ± 4.6 % total phenols removal, 71 ± 1.6 % decrease in colour and increased the pH from 5.3 to near-neutral. Laccase activity in flask cultures peaked at 4644 ± 228 units/l. A variety of reactors were assessed and a bubble-lift reactor was chosen for a large-scale treatment process. A 50 l working-volume, bubble-lift reactor was constructed and autoclaved wastewater was aseptically inoculated with *T. pubescens* MB 89. Problems with aeration led to lower remediation efficiencies, but laccase synthesis was extremely high and peaked at 12966 ± 71 units/l. *Trametes pubescens* MB 89 greatly improved the quality of the brandy distillery wastewater while simultaneously producing high concentrations of an industrially relevant enzyme.

After the promising results in Chapter 4, more wastewaters were obtained from distilleries and wineries in the Western Cape and their bioremediation by *T. pubescens* MB 89 was examined. In Chapter 5 six high-strength wine-related wastewaters were partially characterised and inoculated with *T. pubescens* at different wastewater concentrations in order to assess remediation and enzyme production by the white-rot fungus. The wine-related wastewaters consisted of a wine lees, two brandy distillery wastewaters and two wastewaters resulting from the distillation of ethanol from low-grade wines and a distilled wine lees that had had its tartaric acid extracted. Crudely purified laccase was tested independently to

determine its role in phenolic compounds degradation and colour change. The pH of these wastewaters was adjusted to 4.5; although this pH was slightly lower than optimal for fungal growth it was the highest pH value at which laccase from *T. pubescens* MB 89 was still catalytically efficient, and allowed for comparison between the fungal and enzymatic treatment systems. The complete fungal system was found to be superior to enzymatic treatment alone. Enzymatic treatment reduced the concentration of total phenolic compounds, but did little to improve the colour of the wastewaters. In the case of wine lees the laccase treatment significantly increased the colour, by up to 160 ± 5 %, indicating the formation of colour-rich compounds, even though it lowered total phenolics by up to 61 ± 0.5 %. The fungal treatment resulted in decreases in the wastewaters' COD of up to 83 ± 2.1 %, phenolic compounds of 87 ± 1.6 % and colour of 88 ± 4.7 %. Although *Trametes pubescens* MB 89 greatly improved the quality of all six wastewaters tested, two wastewaters had to be diluted to below 50 % to allow for remediation by the submerged fungal culture. Laccase synthesis greater than 1500 units/l was obtained in all wastewaters, with a maximum of 8997 units/l in the brandy distillery wastewater.

Chapter 6 characterises and assesses the treatment of a distillery wastewater unique to southern Africa, generated from Amarula production, using *T. pubescens* MB 89 in various wastewater concentrations at pH 4.5. In shake-flask cultures *T. pubescens* MB 89 attained 71 to 77 % COD removal, 87 to 92 % total phenolic compounds removal, and an increase in pH for all concentrations tested. The fungal culture could treat the wastewater at full strength, requiring only a small increase in pH. The colour decreased for samples below 60 % wastewater concentration, while it increased for the 80 and 100 % concentrations. Maximum laccase activity obtained was 1063 ± 26 units/l after fourteen days in the 80 % wastewater concentration. This is the first known work published regarding Amarula wastewater characterisation, bioremediation and the synthesis of a valuable by-product as a result of fungal treatment.

Trametes pubescens MB 89 was assessed for its robustness as a potential remediator of widely variable winery wastewaters and its value as a pre-digestion treatment step in Chapter 7. Sixteen different winery wastewaters were collected during peak wine production (March

2006) and were characterised in terms of pH, concentrations of total phenolic compounds, COD, colour, nitrite, ammonia, nitrate, phosphates, chloride and presence of lactose or non-lactose fermenters. Fungal treatment of the raw, sterilised wastewaters decreased the COD and increased the pH of all samples. Total phenolic compounds decreased in all samples with an initial concentration greater than 20 mg/l, while those originally below 20 mg/l were elevated slightly after fungal treatment. The decrease in the concentration of total phenolic compounds correlated to the decrease in colour. A slight increase in colour was observed for the samples that were originally very low in colour. Five of the samples displayed an increased synthesis of laccase, but it was very low (less than 400 units/l) and could not be related to any of the individual parameters tested. Anaerobic digestion comparing fungally-pre-treated and raw samples generally showed little difference with regard to total COD removal and final pH. For samples having higher initial total phenolic compounds and colour concentrations it proved advantageous to pre-treat the wastewater samples using *Trametes pubescens* MB 89, while it proved disadvantageous for samples with lower initial total phenolic compounds and colour concentrations.

Chapters 3 to 7 had shown that *T. pubescens* MB 89 could be used to remediate a variety of distillery and winery wastewaters. The synthesis of laccase by *T. pubescens* was focused on in Chapter 8. Several parameters were assessed to determine favourable conditions and growth medium constituents that would maximise laccase synthesis by *Trametes pubescens* MB 89. It was hoped that adjustment of certain parameters would enable consistently high production of the enzyme. Various growth conditions, including pH, carbon sources, nitrogen sources and cellulosic additions were tested to maximise laccase synthesis. A pH value of 5.0 yielded the highest laccase activity of the values tested. A number of potential inducers were then assessed under optimised growth conditions with addition prior to inoculation and in the exponential growth phase. Ethanol, copper and 2, 5-xylidine added prior to inoculation were found to be the best inducers. A new approach of regular dosage of 2, 5-xylidine during the growth phase was found to maximise laccase synthesis. This contrasted with prior literature, where the inducer was added once, either prior to inoculation or when the inoculum was actively growing (three or four days after inoculation). Some growth medium constituents and inducers were tested in three distillery wastewaters and a wine lees to determine which would

enhance laccase synthesis. A synergistic effect was observed with the addition of glucose, copper and 2, 5-xylidine and it notably increased laccase synthesis in flask cultures of all four wastewaters. Glucose repression was countered by 2, 5-xylidine and copper addition as laccase synthesis was greatest during the initial growth period. No individual factor resulted in enhanced laccase synthesis in all four wastewaters tested.

This work showed that a number of varying wastewaters could be remediated by *Trametes pubescens* MB 89 and that high concentrations of a valuable enzyme could be produced concurrent with remediation. It also showed that there were great differences not only between different wastewaters, but also between separate batches of the same type of wastewater.

Chapter 2

Literature review, hypothesis and objectives

2.1 Literature review

2.1.1 Introduction to distillery and winery wastewaters

A great volume of wastewater is generated daily by processes involved in the distillation of fermented sugar sources to obtain ethanol. The global production of ethanol in 50 countries was in the region of 51.1 million litres in 2006. The U.S.A. was the largest producer. It produces nearly 20.1 million litres of ethanol per annum, mainly from the fermentation from corn, while Brazil produces 17.0 million litres per annum, mainly from sugarcane. Together they produce more than 70 % of the world's ethanol. The rest of the world produces approximately 14 million litres (Jackson, 2007). Figures for the annual volumes of wastewater produced are difficult to obtain, but the molasses-based distilleries generate eight to fifteen litres of wastewater for every litre of ethanol produced (Nataraj *et al*., 2006). In India alone 285 distilleries generated 40×10^6 m^3 of wastewater in 2004 (Raghukumar *et al*., 2004). By July 2004 more than 4000 distilleries were listed in F.O. Licht's World Distilleries Guide 2004/5, spanning 144 countries. At the most modest of assumptions the global wastewater generated from ethanol production is in the region of 410×10^6 m^3 per annum. This volume is predicted to rise astronomically over the next five years due to the demand for fuel ethanol. The problem of increasing volumes of distillery waste has been long anticipated. An excerpt from a paper written more than a quarter of a century ago by Sheehan and Greenfield (1980), states that: "The volume of stillage will increase drastically in those countries which opt for ethanol from biomass as a liquid fuel supplement. This will create severe local water pollution problems unless treatment is enforced. The costs of this treatment are likely to be of the same order of magnitude as the fermentation costs themselves". This is particularly relevant now that easily exploitable oil reserves will be depleted before the next century and alternatives such as biofuels are being intensely researched, funded and subsidised by governments and private investors.

Wineries and other grape processing industries also generate large volumes of wastewater annually. These are derived from cellar wastes as well as distillation wastewaters for the production of brandy or ethanol. The winery wastewaters mainly originate from various washing operations during the crushing and pressing of grapes, as well as rinsing of fermentation tanks, barrels and other equipment. South African regulatory bodies dictate that

wastewater discharged into the environment should have a pH between 5.5 and 7.5 and the chemical oxygen demand (COD) should not exceed 75 mg/l (South African Water Act no. 36, 1998). Winery wastewaters typically have an acidic pH between 3 and 4 and a COD of 0.8 to 12.8 g/l. The COD can increase to 25 g/l depending on the harvest load and processing activities. The chemical analysis of winery wastewater has indicated that the high concentrations of sugars make up a large portion of the COD, while organic acids play a more prominent role in the acidity of the wastewater (Malandra *et al*., 2003). Some wineries in urban areas channel the wastewater to local sewage treatment facilities that result in heavy penalties due to the low acid pH and high COD. In contrast, rural wineries often have very little or no treatment operations for wastewater, which is often irrigated onto grass fields. This may eventually have deleterious side effects. An analysis of 21 Spanish winery and distillery wastewaters showed an acidic pH, a high organic load and notable polyphenol, macronutrient, micronutrient and heavy metal contents (Bustamante *et al*., 2005). Some of the properties of these wastewaters were not compatible with agricultural requirements, making treatment necessary in order to produce an environmentally safe, stable and easily manageable wastewater.

A variety of raw materials are fermented and distilled for the production of alcohol for spirits or other uses, including sugarcane molasses, rye, barley and wheat. Distillation industries often produce large volumes of waste that are high in organic strength and are a major source of soil and water pollution (Sheehan and Greenfield, 1980). Wine distillery wastewater, or vinasse, is produced by the distillation of wine, wine lees or fermented grape juice to extract ethanol or some flavour compounds to produce brandy and other spirits. Due to the acidity, colour and high COD of this wastewater it is vital that it is treated prior to discharge, as it will lead to eutrophication and damage of the receiving environment. Wine distillery wastewater is acidic and varies in colour from yellow to dark red due to the phenolic compound content. It also contains compounds such as organic acids, salts, yeast cells, soluble proteins and carbohydrates (Sales *et al*., 1987), which lead to a high biological (biochemical) oxygen demand (BOD) and COD ranging from 11 to 110 g/l (Bustamante *et al*., 2005; Dekker, 2003; Ramana *et al*., 2002a; Borja *et al*., 1993a; Robertiello, 1982). A distillery in Wellington in South Africa uses grape wine feedstock that is fermented and then distilled to separate ethanol

from the fermented liquid. The resulting wastewater stream is highly polluted, with a COD of 20 to 30 g/l and a pH between 3 and 4. Wastewater from a wine distillery consists primarily of organic acids with a high soluble biodegradable COD fraction of 98 % (Moosbrugger *et al*., 1993). The typical composition of a wine distillery waste with respect to organic acids has been found to be 27 % tartaric acid, 8 % malic acid, 29 % lactic acid, 26 % succinic acid and 10 % acetic acid (Wolmarans and de Villiers, 2002). The composition of South African distillery wastewater during the 1999 harvest season displayed the following properties: pH 3.7 to 4.8, COD 7.4 to 28.5 g/l, conductivity 140 to 400 mS/m, total dissolved solids 1000 to 2800 mg/l, nitrates 35 to 132 mg/l, chlorides 20 to 425 mg/l, phosphates 98 to 251 mg/l, ammonia 68 to 378 mg/l, potassium 330 to 490 mg/l, sodium 18 to 170 mg/l, magnesium 14 to 86 mg/l, calcium 21 to 90mg/l (Malandra *et al*., 2004).

A modified extract from the Environment Protection Authority guidelines for wineries and distilleries in South Australia (EPA, 2004) is shown in Table 2.1. This table makes it evident that there are a number of constituents and properties that can adversely affect the environment should the untreated wastewater be discharged.

Table 2.1: Potential environmental impact of winery and/or distillery wastes (adapted from EPA, 2004).

Constituent	Indicators	Effects
Organic matter	Biochemical oxygen demand, total organic carbon and chemical oxygen demand.	Depletes oxygen when discharged into water leading to the death of fish and other aquatic organisms. Odours are generated by anaerobic decomposition if waste is stored in open lagoons or applied to land. Phenolic compounds may reduce light transmission in water.
Acidity	pH.	Kills aquatic organisms at extreme values. Affects crop growth, microbial activity in biological wastewater treatment processes, solubility of heavy metals in the soil and availability and/or toxicity in waters.
Nutrients	Nitrogen, phosphorus and potassium.	Can lead to eutrophication or algal blooms when discharged to water or stored in lagoons; algal blooms can cause undesirable odours. Toxic to crops in large doses. Nitrogen as nitrate and nitrite in drinking water can be toxic to infants.
Salinity	Electrical conductivity and total dissolved salts.	Imparts undesirable taste to water, is toxic to aquatic organisms and affects water uptake by crops.
Sodicity	Sodium adsorption ratio and exchangeable sodium %.	Affects soil structure (hard and dense subsoil) causing surface crusting, low infiltration and hydraulic conductivity.
Heavy metals	Cadmium, chromium, cobalt, copper, nickel, lead, zinc, mercury.	Toxic to plants and animals.
Solids	Total suspended solids.	Reduces soil porosity and leads to reduced oxygen uptake. Reduces light transmission in water. Anaerobic decomposition generates odours.

Numerous phenolic compounds are present in wines as a result of their extraction from the skin, flesh and seeds of grapes. Phenolic compounds form a relatively small portion of the wastewater COD, but can have great negative effects upon treatment systems as well as environmental damage if released untreated. These compounds have been implicated in the inhibition of biological treatment systems for both winery and distillery wastes. Red wines have much greater and more diverse phenol content than white wines. The amount and type of phenolic compounds extracted, and their extent of polymerisation is the most influential factor determining a red wine style. The basic phenol group is an aromatic benzene ring with at least one hydroxy group attached. A number of compounds containing this highly reactive phenolic group are produced and are extracted from the grapes during wine production.

The types of phenolic compounds in grapes and wines can be broadly categorised into flavonoid and non-flavonoid phenols. Examples and a basic categorisation of the major phenolic compounds are shown in Table 2.2. Unfortunately, some compounds are classified differently according to different authors, making generalisations such as those in Table 2.2 somewhat difficult. The flavonoid phenols are responsible for many of the characteristics of red wine, including colour, mouthfeel and texture, and ageing characteristics, and they mostly occur in the grape skins, seeds and plant stems. Anthocyanins are responsible for the colour of red wines and are involved in the phenolic polymerisation reactions that occur during ageing. The most common flavonols in grapes are quercetin and kaempferol (Price *et al*., 1995). Flavanols are the isomers catechin and epicatechin that make up the procyanidins and condensed tannins of wines. The flavanols are bitter and slightly astringent, but are not classed as tannins as they do not precipitate proteins (Bate-Smith, 1973). Some authors consider the procyanidins to be flavanols, while others consider them as a separate group. Procyanidins (or proanthocyanidins) are usually considered to be the oligomers of two to eight flavanol units, with larger or more complicated structures referred to as tannins. They react with other phenols and anthocyanins, and precipitate with proteins.

White wines are made with minimal skin and seed extraction and therefore contain relatively small amounts of flavonoid phenols. Their phenol content comprises mostly non-flavonoid phenols. Oak contact can also add to the non-flavonoid phenol content of wines by

contributing hydroxybenzoates from lignin degradation, as well as hydrolysable tannins. The non-flavonoid phenols of grapes are primarily hydroxycinnamic acid esters and the hydroxybenzoic acids (gallic, vanillic, *m*-hydroxybenzoic, *p*-hydroxybenzoic and syringic acid). Gallic acid is also found in grape seeds combined with (-)-epicatechin to form epicatechin-3-O-gallate, and it can contribute to the formation of condensed tannins in grapes. Hydroxybenzoic acids may also occur in red wines as degradation products of anthocyanins. The concentrations of hydroxybenzoic acids in red wines are rather low, especially compared to the amounts of other phenols present. Hydroxycinnamic acids (caffeic, coumaric and ferulic acids) are the predominant non-flavonoid phenols in wines (Hernanz *et al*., 2007), and exist in grapes only as tartaric acid esters such as caffeoyl tartaric acid (caftaric acid), coumaroyl tartaric acid (cutaric acid) and ferouyl tartaric acid (fertaric acid) (Gil-Muñoz *et al*., 1999). Although the free forms of these acids do not exist in grapes, they can be found in wines due to enzyme and acid hydrolysis during the winemaking processes (Sarni *et al*., 1995; Macheix *et al*., 1991).

Table 2.2: Table of the major flavonoid and non-flavonoid phenolic compounds found in wines.

Flavonoid phenols			
Flavonols	**Flavanols**	**Anthocyanins**	**Procyanidins (proanthocyanidins or condensed tannins)**
Kaempferol[1]	(+)-Catechin[2+3]	Delfinidin 3-glucoside[1]	Procyanidin B[1]
Kaempferol 3-rutinoside3	(-)-Epicatechin[2+3]	Cyanidin 3-glucoside[1]	Procyanidin B2[1]
Quercetin[1+2]		Petunidin 3-glucoside[1]	Procyanidin B3[1]
Quercetin-3-*O*-glucuronide[2]		Peonidin 3-glucoside[1]	Procyanidin B4[1]
Quercetin-3-*O*-galactoside[2+3]		Malvidin 3-glucoside[1]	Trimer C1[1]
Quercetin-3-rutinoside[3]		Peonidin 3-(3-acetyl glucoside)[1]	
Dihydroquercetin derivative[3]		Malvidin 3-(3-acetyl glucoside) [1]	
Quercetin glycoside[3]		Malvidin 3-(3-coumaryl glucoside) [1]	
Myricetin[1+2]		Pelargonidin	
Myricetin-3-*O*-glucoside[2+3]			
Myricetin 3-galactoside[3]			
Isorhamnetin-3-rutinoside[3]			
Non-flavonoid phenols			
Hydroxycinnamic acids and their derivatives:	**Hydroxybenzoic acids:**	**Stilbenes**	**Other compounds**
Cis[1] *and trans-* Caftaric acid[2]	Gallic acid[2+3]	*cis and -trans-*	Tyrosol[2]
Cis[1] *and trans-* Coutaric acid[2]	Protocatechuic acid[2+3]	Resveratrol[2+3]	Tryptophol[2]
trans- Caffeic acid[2]	*p*-Hydroxybenzoic acid	(3, 4, 5-trihydroxy-	Ethyl gallate[3]
trans- p-Coumaric acid[2]	Vanillic acid[2+3]	trans/cis-stilbene)	Methyl gallate[3]
trans-p-coumaric glucoside[3]	Syringic acid[2+3]	*cis and trans-*	
Ferulic acid	Ethyl gallate[2]	Resveratrol-1-*O*-	
Fertaric acid	Methyl gallate[2]	glucoside[2+3]	

[1]García-Falcón *et al*., 2005.
[2]Monagas *et al*., 2005.
[3]Hernández *et al*., 2006.

Some of these compounds are extremely good antioxidants and can be recovered from some of the waste residues associated with wineries. Resveratrol has been intensively studied due to moderate red wine consumption being associated with lower incidences of coronary disease. Louli *et al*. (2004) investigated the extraction of the antioxidants contained in the pomace of red grapes. Ethyl acetate was found to be the most appropriate solvent due to its low boiling point and its non-toxicity, as well as its extract having the highest antioxidant activity. Antioxidant recovery is very appealing, due to its low cost, the high added value of the recovered phenolic compounds and the high amounts of relatively unexploited by-products resulting from the winemaking procedure.

Many of the compounds present in wine have the potential to occur in wastewaters generated from their distillation. Distillation would lead to thermal degradation or a variety of reactions that degrade or convert the more sensitive compounds to more stable compounds, but a large portion of the more stable, non-volatile, phenolic compounds summarised in Table 2.2 have the potential to be present in waste from distillation of wine for the production of ethanol or brandy.

2.1.2 Treatment of distillery and phenolic-rich wastewaters

Removal of phenolic compounds from industrial wastewaters is an important practical problem, since many of these compounds are toxic and their presence in drinking and irrigation water is potentially hazardous. Conventional purification methods such as solvent extraction, adsorption on activated carbon and chemical oxidation suffer from serious drawbacks such as high costs associated with materials and the formation of by-products that are toxic to the environment (Lante *et al.,* 2000). Many phenolic compounds are not readily biodegradable and are toxic to many microorganisms, even at low concentrations. Some microorganisms are capable of using aromatic compounds as their sole source of carbon and energy, but phenol can inhibit the growth of species that have the ability to metabolise it. Phenol can be toxic or even lethal to fish at relatively low concentrations of 5 to 25 mg/l and it also contributes to off flavours in drinking and food-processing waters. In humans ingestion of phenol produces burning pain and white necrotic lesions in the mouth, oesophagus and stomach, vomiting and bloody diarrhoea. After skin exposure, pain is followed by numbness

and the skin becomes blanched. The systemic clinical effects of phenol include headaches, dizziness, hypotension, ventricular arrhythmia, shallow breathing, cyanosis, pallor and initial convulsions followed by unconsciousness. A fall in body temperature and pulmonary oedema may occur. The most important effects in short-term animal studies are neurotoxicity, liver and kidney damage and respiratory effects (Inder, 1997).

Numerous methods have been published to treat or dispose of the phenolic-rich wastes that originate from wineries and distilleries. Physical and chemical processes include filtration (Nataraj *et al*., 2006), oxidation by ozone (Amat *et al*., 2003), chlorine dioxide, hydrogen peroxide and radiation (Benitez *et al*., 2003) and adsorption to compounds such as chitosan (Lalov *et al*., 2000) and activated carbon. Biological treatment processes include aerobic digestion, which can utilise activated sludge (Petruccioli *et al*., 2002), rotating biological contactors (Malandra *et al*., 2003), shallow ponds and trickling filters (Travieso *et al*., 2006) and anaerobic digestion, which can utilise methanogenic bacteria to produce biogas (Nandy *et al*., 2002). Extensive treatment systems such as wetlands (Billore *et al*., 2001), ponding and land application (Ryder, 1995) make use of combinations of these processes concurrently. Combinations of these methods have also shown positive results in intensive processes (Bazua *et al*., 1991). Dual stage processes may have an initial stage that removes the toxic components of the wastewater, which allows for better degradation kinetics in a second stage designed to remove the majority of the COD. Alternatively, the step removing the majority of the COD occurs first, and this is followed by a subsequent polishing step to obtain the desired discharge parameters.

Although anaerobic digestion has the ability to treat high organic loads, the phenolic compounds, salt ions and sulphides present in the wastewater may inhibit the treatment process. Wine and fruit industry wastewaters are not ideal for anaerobic digestion due to their low nitrogen and phosphorus concentrations and the seasonal variation (leading to inconsistent loading of digesters). The advantages of anaerobic digestion are production of methane gas as a by-product and decreased biomass production. Most aerobic systems are effective at COD reduction provided there is adequate aeration, but entail high capital requirements and running costs.

2.1.3 Physicochemical treatment processes

A variety of physicochemical methods have been shown to decrease the pollutant load of distillery and winery wastewaters. These include membrane filtration, oxidation by ozone, chlorine dioxide, hydrogen peroxide and radiation and adsorption to materials such as chitosan and activated carbon.

Ozone is a powerful oxidant and can be used to oxidise compounds present in wastewaters that may be toxic to microorganisms. At low pH, ozone exclusively reacts with compounds with specific functional groups through selective reactions (Langlais *et al*., 1991), while at a basic pH, ozone decomposes to yield hydroxyl radicals (Legrini *et al*., 1993), which oxidise a wide range of organic and inorganic compounds non-selectively in water. Ozone and chlorine dioxide were tested as oxidants to eliminate gallic acid and epicatechin from distillery wastewaters. Both oxidants were found to have a similar capacity to eliminate both organics, although chlorine dioxide oxidation was found to be faster. This was attributed to the mass transfer rate of ozone transfer hindering the oxidation rate (Beltran *et al*., 1993). The oxidation of distillery wastewaters with UV radiation and combined with hydrogen peroxide was investigated by Beltran *et al*. (1997a). Distillery wastewaters were shown to be refractory to UV radiation, although the addition of hydrogen peroxide led to COD removal. Beltran *et al*. (1997b) then compared distillery wastewater oxidation using ozone combined with hydrogen peroxide or UV radiation. The addition of hydrogen peroxide resulted in a minor increase in the oxidation rate. However, the combination of ozone and UV radiation was found to improve COD and total organic carbon degradation rates compared to ozonation alone.

Beltran *et al*. (2001) diluted wine distillery wastewater with domestic wastewater and found pH sequential ozonation advantageous compared to ozonation at constant pH at COD removal (24 % versus 19 % removal), but that ozonation at a constant pH led to higher removal of polyphenols (33 % versus 19 % removal) and UV_{254} absorbing compounds. A three to five minute ozonation was found to be optimal to increase biodegradability more than ten times using solutions containing a mixture of cinnamic acid, *p*-coumaric acid, caffeic acid and

ferulic acid. The enhanced biodegradability was attributed to the formation of highly biodegradable benzaldehydes as key ozonation intermediates (Amat *et al*., 2003).

Benitez *et al*. (2003) later studied the effect of ozonation, hydrogen peroxide and UV treatment on winery wastewater and the subsequent aerobic digestion of the pre-treated wastewater. The single ozonation process led to 5.0 to 25.2 % removal of COD and 16.8 to 51.4 % removal of the total aromatic compounds. They found that ozonation prior to digestion by the activated sludge improved substrate removal from 27.7 to 39.3 %. Digestion by an activated sludge system removed 31 to 85 % of the COD with a hydraulic retention time (HRT) between 24 and 72 hours. They attributed the improved organic matter removal and kinetic parameters to the contribution of the hydroxyl radicals generated in the combination of UV radiation, hydrogen peroxide and ozone. Carbajo *et al*. (2007) assessed the enhanced catalytic degradation of compounds in one synthetic and three real wastewaters using ozonation combined with perovskites (a calcium titanium oxide mineral). The synthetic (a mixture of syringic, pyruvic and gallic acids in water), wine distillery, olive debittering, and olive oil production wastewaters showed different behaviours depending on their nature. No differences were observed between catalytic and non-catalytic ozonation of easily oxidised wastewaters, but the addition of perovskites enhanced the ozonation process for wastewaters that were refractory towards a single ozonation treatment.

Photocatalytic advanced oxidation is a promising technology for waters containing high amounts of organic matter. Navarro *et al*. (2005) investigated the application of H_2O_2 as oxidant combined with light (artificial or natural) in order to reduce the organic matter in samples from wine industry wastewaters. When H_2O_2 was combined with heterogeneous catalysts (titanium dioxide and clays containing iron minerals) less H_2O_2 was required. The combination with titanium dioxide produced higher efficiencies, but three and six times less H_2O_2 was required when using clays to enhance photocatalysis. Removal of organic compounds by ionising radiation occurs due to various reactions. These reactions include combining with the primary products of water radiolysis, secondary short-lived species, as well as secondary processes, such as the formation of adsorbent precipitates (Pikaev, 2002). Pikaev *et al*. (2001) combined electron-beam (ionising radiation) and coagulation treatment to

purify molasses distillery slops from distillery produced ethyl alcohol by fermentation of grain, potato, beet and some other plant materials that were mixed with municipal wastewater. It was estimated that a plant purifying 7000 m^3/day would cost approximately US$ 0.25/m^3 of wastewater.

Yavuz (2007) investigated the electrochemical treatment of alcohol distillery wastewater using an iron electrode as well as the effects of adding hydrogen peroxide. He obtained a COD removal efficiency of 92.6 % (initial COD of 4985 mg/l) and TOC removal efficiency of 88.7 % (initial TOC of 1507 mg/l) for the pre-treated alcohol distillery wastewater. Yavuz (2007) also tested electrocoagulation, but found it to be ineffective under all experimental conditions studied. Piya-areetham *et al*. (2006) had previously conducted laboratory scale experiments to reduce colour and COD in distillery wastewater using electro-oxidation processes. They found that maximum oxidation of organic pollutants in wastewater occurred under acidic conditions and the presence of additives promoted oxidation to attain a COD reduction of 89.6 % and colour reduction of 92.2 %.

A hybrid nanofiltration and reverse osmosis pilot plant was successful for the removal of colour-containing compounds as well as 99.8 % total dissolved solids, 99.9 % of COD and 99.99 % of potassium from distillery spent wash, while the membranes were not affected by fouling (Nataraj *et al*., 2006). A large amount of water could be permeated economically (as opposed to being vaporised by energy intensive evaporation processes or steam distillation using the tall towers) and reused for either municipal or industrial purposes. An advantage of recycling wastewater that had been treated by reverse osmosis had been reported much earlier by Kato *et al*. (1977). Wastewater recycled back into the corn mashing stage increased the ethanol yield by 5.8 % compared to using fresh water.

Lalov *et al*. (2000) described the purification of vinasse-containing water by an anion-exchange process using chitosan. The positive charge allowed successful adsorption of the main acid components of vinasse. Chitosan is a modified, natural, carbohydrate polymer derived from the chitin component of the exoskeleton of crustacean such as shrimp, crab, crawfish etc. Chitosan's non-toxicity, biodegradability, flocculating ability, polyelectrolisity

and possibility for regeneration have led to a number of applications. Model experiments have shown a chitosan concentration of 10 g/l and a contact time of 30 min to be the most suitable for the purification of the model wastewater with a COD of 2800 mg/l. Subsequent biomethanation of adsorbed organic acid anions on the ion exchanger by a direct addition of chitosan to an anaerobic bioreactor before and after preliminary hydrolysis was also demonstrated. The costs involved maintaining a chitosan suspension for maximal removal efficiency and separation of the spent chitosan to prevent subsequent clogging of anaerobic digesters would make this apparent product recovery exceedingly impractical.

Chaudhari *et al*. (2007) assessed inorganic coagulants (aluminium chloride, ferric chloride and polyaluminium chloride) for the removal of molasses-derived coloured compounds and COD from the biodigester wastewater of a molasses-based alcohol distillery wastewater treatment plant. Initial flocculation may be a better alternative to the conventional aerobic treatment process for the removal of recalcitrant COD. The treatment resulted in 55, 60 and 72 % COD reductions and 83, 86 and 92 % colour reductions using aluminium chloride, ferric chloride and polyaluminium chloride, respectively, at their optimum initial pH. Although the filtration characteristics of the flocculated wastewater were poor the solid residue (obtained by filtration and drying of the polyaluminium chloride treatment) had a specific energy of 13.4 MJ/kg and could be used as a medium energy fuel material.

Chemical and physical removal methods have been shown to work well, but also have high costs associated with initial capital investment, raw materials and removal of solids from waste treatment process. Although these methods have an inherent advantage in that they are not reliant on living organisms and there are potential methods of energy recovery, there are serious shortcomings in both practical implementation and financial feasibility. These shortcomings include high capital investment and specific requirements for reaction conditions such as pH and mixing. They may also require filtration to remove suspended solids and may generate waste in the form of flocculated compounds or spent adsorptive materials.

2.1.4 Extensive treatment systems

According to Mulidzi *et al*. (2002), more than 90 % of wine cellars in South Africa dispose of their wastewater by means of land application, mainly irrigation. According to a survey conducted by Sheridan *et al*. (2005), 60 % of wineries dispose of their wastewaters by irrigation, 10 % discharge it into municipal drains, 10 % is evaporated, 7 % treat their waste with French drains, while the remaining 10 % of wastewater is treated by dams, storage dams, wastewater treatment plants, river discharge or other, unspecified means. Land application was considered to be the most effective and practical disposal method for water, nutrient and organic matter contents of winery wastewater (Ryder, 1995). Tano *et al*. (2005) compared increasing doses of distillery vinasse factorially combined with three levels of urea on vegetative growth, leaf mineral levels, grape yield and quality over a four year period. Both vinasse as a nitrogen source and ureic nitrogen reduced the number of blind buds and increased the potential and actual bud fertility, but no additive effects were noted. The highest level of vinasse that was applied improved the ripening levels of grapes, increasing sugars and reduced grape juice acidity; indicating the possibility of reuse of vinasse within the vineyard. However, Juwarkar and Dutta (1990) found raw distillery wastewater highly toxic to the soil microorganisms, which are important in the soil ecosystem. A pot culture experiment found raw wastewater application decreased the population of bacteria, fungi and actinomycetes while the growth rates of *Rhizobium* and *Azotobacter* were reduced. Mixing of the raw wastewater with stabilisation pond wastewater (1:1) minimised toxic effects as was demonstrated by an increase in the populations of all the microorganisms studied. Raw wastewater toxicity was further corroborated when used for irrigation in a groundnut plant; no fruits were produced and less nodulation was observed.

Winery wastewaters pose severe pollution problems, mainly because the organic component leaches down to the water table where decomposition is slow, due to the shortage of oxygen. Unpleasant odours may also be released if the soil is disturbed. The organic material is mobile in the groundwater and constitutes a major off-site pollution hazard. Large volumes of wastewaters are often discharged on small areas of land, which aggravates leaching (Mulidzi *et al*., 2002). Another major potential problem with land application is that of inorganic ions. These can lead to the degradation of soil fertility and structure in the longer term. Root zone

accumulation of salt must also be avoided as it may negatively affect plant growth. Land application must be adequately monitored to protect water resources from the cumulative effect of many small additions (Bowmer and Laut, 1992).

Generation of products from wastes can coincide with waste treatment, even while using extensive treatment systems. Berri Estates' winery in Australia has been using the wastewater from its winery for intermittent irrigation of a plantation of Murray River Redgums (*Eucalyptus camaldulensis*). The highly seasonal wastewater could not be treated easily by traditional lagooning systems and had associated odour problems. The winery produced around 200 megalitres (BOD: 2.5 g/l) of wastewater annually, while the distillery produced 10 megalitres of wastewater (BOD: 15 g/l). Irrigation of the wastewater occurred at fourteen day intervals (limited by evaporation, transpiration and infiltration). By controlling the loadings and penetration depth, anaerobic conditions in the soil and thus odour production were prevented (AGDEWR, 2001).

Ramana *et al*. (2002a) conducted a laboratory experiment to determine the effect of different distillery wastewater concentrations on tomato, chilli, bottle gourd, cucumber and onion seeds. Although the species displayed differing sensitivities to wastewater, complete failure of germination was observed once the wastewater concentration used for irrigation exceeded 50 %. The speed of germination, peak value and germination value also followed similar trends. The effect of the distillery wastewater was crop-specific and care was advised when using distillery wastewater for presowing irrigation purposes. The observed variation in seed germination of crops could have been attributed to excessive quantities of inorganic salts. Unfortunately fungal growth on the seeds with concentrations greater than 25 % may also have been one of the factors contributing to poor germination at higher wastewater concentrations. Other work done with maize (Ramana *et al*., 2002b) and groundnut (Ramana *et al*., 2002c) suggests that distillery wastes should not be used in isolation, but could serve well as a supplement to lower fertilizer requirements.

Lagoon or pond treatment has the advantage of being a relatively low maintenance method of wastewater treatment technology and is feasible if the land is available, inexpensive and not

situated close to human habitation. Often extensive methods are not financially viable as distilleries or wineries are located in regions where the land is intensively used for crop production and therefore expensive. If the distillery is located in an urban area land is rarely available or cheap and associated odours arising from anaerobic digestion can lead to heavy penalties. Rao (1972) treated wastewater in a 1.8 m deep anaerobic lagoon followed by a 0.9 m deep aerobic finishing lagoon. The first lagoon achieved BOD removals ranging from 95 to 55 %, but required long HRTs, of 38 to 66 days. When combined with the aerobic lagoon the overall BOD removals were between 92 to 84 %, but this included an additional 43-24 days HRT. The largest Indian distillery, Hindustani Sugar Mills Ltd, treated stillage combined with sugar refinery wastewater (BOD: 16 g/l) in lagoons. Three hectares of the lagoons were 2-3 m deep, 2 ha were 2 m deep, and the final 2 ha were 0.9-1.0 m deep. Total volumetric flowrate of the influent was 800 m^3/day and overall 88.8 % BOD was removed with a HRT of 18 to 20 days. The treated wastewater had a BOD of 1800 mg/1 and was diluted and used for irrigation (Sheehan and Greenfield, 1980). In California multi-stage, facultative aerobic ponds have been used successfully for treatment and storage of winery wastewater for more than 40 years (Ryder, 1995). Ponds are lined to prevent seepage into the water table and aeration prevents the generation of malodorous compounds. The treated water is utilised for irrigation during periods of low rainfall. Bories *et al*. (2005) studied the origin of malodorous compounds and methods to prevent and treat odour formation during treatment of winery and distillery wastewater by natural evaporation in ponds. When nitrate (an electron acceptor) was added, catabolism led to the oxidation of the organic compounds to CO_2 and the nitrate was reduced to N_2 (an odourless gas), without volatile fatty acid (VFA) formation. They tested nitrate addition on an industrial scale in winery and distillery ponds and results showed that it was possible to decrease pond odours with nitrate addition.

It is possible that constructed wetlands may allow for effective, low maintenance purification of distillery and winery wastewaters. Although they are land intensive treatment systems, wetlands provide many benefits which include water quality improvement, food and habitat for wildlife, flood protection and shoreline erosion control. Constructed wetlands are engineered systems that have been designed to make use of macrophytic vegetation, soils and a variety of aerobic and anaerobic bacterial populations and fungi and yeasts to remediate

wastewaters. The macrophytic vegetation can be classified as emergent, submerged, free-floating or rooted plants with floating leaves. The flow regime is important in the classification of a wetland, as it can be surface, subsurface, vertical or horizontal. Wetland treatment is advantageous to lagooning in that subsurface flow (through a permeable medium) allows for odour control. However, surface flow simulates wetlands and is sometimes more economical than subsurface flow. A variety of processes such as sedimentation, filtration, precipitation, sorption, plant and microbial nutrient uptake allow for removal of organic materials from wastewaters. Constructed wetland systems can vary from a single-cell to multiple-cells. Appropriately designed constructed wetlands have a large potential application in the treatment of wastewaters. Three to four decades ago constructed wetlands were primarily built to treat domestic or municipal sewage, but in the past 15 years numerous diverse applications have been shown. Leachate or runoff waters (urban, highway and agricultural), food processing wastewaters (resulting from wine, cheese and milk production) and even industrial wastewaters (e.g. chemicals, paper mill, oil refineries and mine drainage) have all been treated using wetlands (Vymazal, 2005). Billore *et al*. (2001) studied a field-scale, four-celled, horizontal subsurface flow constructed wetland for the treatment of a molasses-based distillery wastewater. Although the COD was reduced by 64 % (from 8420 to 3000 mg/l) the wastewater strongly impacted on plant morphology, aeration anatomy in the chiselled plant tissues, reed growth and composition of the biofilm in the specialised substratum. However, when new plant growth emerged eight months later it appeared healthy. Shepherd *et al*. (2001) combined a subsurface-flow constructed wetland with an upflowing sand prefilter and measured average removal efficiencies of 98 % for COD, 97 % for total suspended solids and 78.2 % for nitrogen in winery wastewater. In addition, removals of sulphide (98.5 %), *o*-phosphate (63.3 %), VFAs (99.9 %), phenols (100 %), tannins and lignins (77.9 %) and settleable solids were observed and the acidic pH was neutralised. One disadvantage is that the entry of solids such as pomace and lees into the wastewater system can be lethal to a constructed wetland. A smaller operational winery wastewater constructed wetland that had used suspended solids removal as well as aeration as methods for pre-treatment indicated complete organic load removal from the winery wastewater (Grismer *et al*., 2003).

Although wetlands do allow for a relatively low maintenance and good removal of COD and phenolic compounds, they are not without their problems. The most obvious is the cost of land associated with the spatial requirement for such a treatment system. Another is the gradual sedimentation of the wetland. Black sediment formed from the precipitate of polymerised phenolic compounds is recalcitrant, and during peak season forms far quicker than it can be degraded. It would have to be physically removed to prevent clogging of the artificial wetland. Direct land disposal has relatively low running costs but is dependent on the volume and characteristics of the wastewater produced and land availability. Ponding too has problems with spatial requirements and odours released from anaerobic digestion in the lower sediment layers. Extensive treatment systems generally do not result in direct generation of a saleable by-product from the wastewater treatment process.

2.1.5 Intensive biological treatment and hybrid treatment processes

According to Malandra *et al*. (2003), biological wastewater treatment processes are based on the use of three types of microbial aggregates: static biofilms (e.g. trickling filters), particulate biofilms (e.g. upflow anaerobic sludge blanket reactors) and flocs (e.g. in activated sludge processes). These processes may be aerobic, anaerobic or combinations of both and may even include a physicochemical treatment step. Additionally, the biological systems may be combined with a physical/chemical process to enhance the efficiency of as well as the kinetics of the treatment process.

An example of a combination of a physical and biological treatment system is given by Ng (1986). An activated carbon bed (0.85-1.00 mm diameter) was combined with a suspended aerobic biological system to degrade phenol (50 mg/l), *o*-cresol (50 mg/l), *m*-cresol (50 mg/l) and *p*-nitrophenol (50 mg/l) in a synthetic growth medium. The system was found to be stable when treating feed with a COD ranging from 460 to 688 mg/l. Total phenol removal was 2.34 g/(l.day) and although no phenolic compounds were detectable, the biomass COD removal rate was still adversely affected by the phenol exposure. The carbon bed buffered the microorganisms in the bioreactor from the full exposure of the toxic compound, allowing for a more gradual acclimatisation. A period of COD removal efficiency decline was observed following each subsequent addition of a phenolic compound, but the microorganisms were

also able to adapt to the presence of each new phenolic compound within a shorter period of time. Thirty two days were required to recover from phenol, but only 17, 16 and 8 days were required to recover from exposure to *o*-cresol, *m*-cresol and *o*-nitrophenol, respectively.

Enzymatic pre-treatment of wastewaters has been shown to increase the degradation efficiency of subsequent biological treatment processes. Batchwise methanogenic treatment of pot ales of malt whisky was found to be less efficient than treatment of grain spirit and this was attributed to the amount of dextrin present. Enzymatic hydrolysis shortened the treatment time by more than 25 %. This was taken a step further by aerobically cultivating Koji mould in the pot ale of malt whisky as a substitute for enzymatic hydrolysis. The culture supernatant was treated continuously using a novel upflow anaerobic filter process reactor. A maximum loading rate of 10.8 g/(l.day) was achieved (corresponding to a treatment time of 18 hours) and all residual dextrin was decomposed (Tokuda *et al*., 1998). Sangave and Pandit (2006a) studied enzymatic hydrolysis of alcohol distillery spent wash to ascertain whether it improved aerobic biological oxidation. Their study indicated that enzymatic pre-treatment of the wastewater with cellulase doubled the rate of the subsequent aerobic oxidation when treated at the original pH of 3.8. The rate of aerobic oxidation was increased marginally to 2.3-fold when enzyme pre-treatment was maintained at pH 4.8. Combining ultrasound and enzymatic pre-treatment yielded the best COD removal efficiencies during aerobic oxidation relative to the other combinations tested for the treatment of the distillery wastewater. The initial oxidation rate was four times better than untreated wastewater. It was shown that the rate of the aerobic oxidation was significantly affected by the method of pre-treatment (Sangave and Pandit, 2006b).

Aerobic sludge treatment systems have also been assessed as post-treatment polishing steps. Costa Reis and Santanna (1985) assessed the performance of an aerobic submerged bed reactor (ASBR) as a polishing step for wastewater that had the majority of the COD removed by anaerobic treatment. The ASBR operated continuously for 200 days with HRTs varying from 23 to 4.5 hours, treating diluted alcohol distillery stillage with a COD of 3000 to 3500 mg/l. The ASBR removed 60 to 80 % COD with a 10 to 16 hour HRT. The HRT had been reduced by maintaining high biomass concentrations. Uzal *et al*. (2003) conducted

experiments in shake-flask cultures to determine the potential of aerobic microbes to treat malt whisky wastewater after anaerobic treatment, in order to meet discharge standards. The aerobic digestion led to a further removal of 55 % of the COD and 70 % of BOD after 15 days. The overall anaerobic/aerobic sequential system treatment removed 99.5 % of the COD and 98.1 % of the BOD and was efficient with influent COD concentration up to 34 g/l. Beltran de Heredia *et al*. (2005) used biological aerobic degradation as an initial treatment and combined it with a secondary chemical oxidation step. Wine distillery wastewaters were digested aerobically and then further treated by oxidation using Fenton's reagent. The biological degradation decreased the COD between 75 and 94 %, and the total phenolic compounds concentration by 54 to 79 %. The secondary treatment using oxidation by Fenton's reagent removed 50 to 80 % of the COD and consistently removed more than 90 % of the phenolic compounds from the pre-treated wastewater.

Petruccioli *et al*. (2002) used a 15 l (working volume) jet-loop activated sludge reactor for the aerobic treatment of winery wastewater continuously for more than a year. They treated wastewaters from different wineries that were collected at different periods of the year. Chemical oxygen demand values ranged from 0.8 to 12.8 g/l while loading rates ranged from 0.4 to 5.9 g COD/(l.day) with HRTs that varied from 2.1 to 4.4 days. The system reacted well to sudden variations of loading. The COD removal efficiency obtained was always higher than 90 % (after the initial start up period of 20 to 30 days) and final wastewater COD values ranged between 0.11 and 0.3 g/l. Although the aerobic treatment of winery wastewater using jet-loop reactors was found to be technically feasible, settleability required improvement, even though it was often within acceptable limits. When the microbial population was characterised after 185 days of continuous operation, the majority of the isolates were found to belong to the genus *Pseudomonas.* Many of its species are known for their ability to degrade complex molecules such as polyphenols, to remove phosphorus, for their strong denitrifying activity and for the production of exo-polysaccharides. *Bacillus* spp. were also present as well as large amounts of *Saccharomyces cerevisiae*. Yeast-like fungi, such as *Trichosporon capitatum* and *Geotrichum peniculatum*, were also present both in the treated wastewater and in the biofilm.

Biofilms with natural, mixed populations are used extensively when treating large volumes of dilute wastewaters. Rotating biological contactors (RBCs) are generally easy to operate, require short start-up times, little maintenance, are effectively oxygenated and display little sloughing of biomass (Nicolella *et al*., 2000). Malandra *et al*. (2003) demonstrated a 250 l prototype RBC to be effective for the treatment of winery wastewater as well as wastewater from a bottling plant. Cellar wastewater had its COD reduced by 43 % with an average HRT of one hour (influent COD of 3 to 9 g/l) over a period of three months. The system was subsequently evaluated at a full scale bottling plant and resulted in an average COD removal efficiencies of 34 % and a pH increase of 0.83 units with a HRT of one to four hours. Although thick biofilms developed on the RBC discs, the system was ineffective for the treatment of distillery wastewater. It was suspected that although the microorganisms were able to survive, their metabolic inhibition was attributed to one or more components of the wastewater such as the organic acids or polyphenols. Although the overall COD removal was not very high the RBC displayed potential as a polishing step or for pre-treatment when COD values were high. The RBC could therefore be an effective primary treatment system to lower the COD to more acceptable levels during the peaks of high COD and acidity in the harvest season for treatment by constructed wetlands or other treatment processes.

Bazua *et al*. (1991) described the treatment of sugarcane molasses vinasse at laboratory and pilot scale in a hybrid system that combined an anaerobic fluidised-bed reactor with two aerobic biodisc reactors. High loading rates (up to 34 g COD /(l.day) at short HRTs (as low as two days) were reached in the anaerobic reactor while removing up to 70 % total COD. A by-product in the form of biogas was produced at 6 l/l fluidised bed/day and had a methane content of around 70 to 85 %. The aerobic reactors had a fixed two-day HRT. A biomass yield of 1.8 kg wet biomass per kg of COD removed was obtained. The biomass contained 18 to 30 % crude protein and an appreciable amount of nitrogen, which could have served as a possible protein source for animal feeds.

Another potential method to treat wastewater is with immobilised biomass in a trickling filter system. Trickling filters are similar to RBCs in that they are generally used as a pre-treatment prior to the method that is used to remove the majority of the COD or they are used to polish

the wastewater from that process. They are less expensive to construct, operate and maintain and less sensitive to shock loads than activated sludge units, but do have associated problems. A review by Sheehan and Greenfield (1980) noted that trickling filter units were used as early as 1949 to treat a 1 % rum stillage mixed with domestic sewage. It was observed that spent wine stillage with a COD greater than 20 g/l inactivated trickling filters with a recycle ratio of 4:1 (even at 100:1 dilution with domestic sewage). Problems of fungal growth, anaerobic conditions and *Psychoda* fly growth have been encountered. The odour problem was alleviated by continual rather than batch removal of solids from the final clarifier. Most of the modern literature available deals with domestic waste treatment only. There is little modern scientific literature demonstrating any pertinence of trickling filters to winery or distillery wastewater remediation other than Travieso *et al*. (2006), who incorporated a trickling filter into a three-stage treatment process. Travieso *et al*. (2006) combined a laboratory-scale anaerobic filter with an aerobic trickling filter process terminating with a stabilisation pond to treat wastewaters. Total COD and BOD removals of up to 54 % and 74 %, respectively, were obtained for the most concentrated influent used at 30 days HRT in the stabilisation pond. The trickling filter treatment step reduced the anaerobic filter-treated wastewater's total COD by a further 34 %, the BOD by 28 %, total solids by 33 %, total volatile solids by 32 %, total suspended solids by 25 %, volatile suspended solids by 12 % and increased the pH from 6.5 to 8.2. The trickling filter was operated at steady-state conditions with an organic loading rate of 1.57 g COD/(l.day).

2.1.6 Fungal treatment of distillery and agro-industrial wastewaters

Using fungi to treat wastewaters is not a new concept. Yeasts and spore-forming fungi such as *Aspergillus* and *Penicillium* spp. have been utilised in mixed consortia in the earliest of heterotrophic treatment systems. Quinn and Marchant (1980) carried out early continuous culture experiments using *Geotrichum candidum* in a 350 ml glass column fermenter. When grown in batch and in single and two-stage continuous laboratory culture, reductions of up to 92 % in the BOD of Irish malt whiskey distillery waste were achieved. Maximum COD and total organic carbon reductions were 81 and 76 % respectively and yields of fungal biomass of over 30 g/1 were obtained. Later, Kida *et al*. (1995) pre-treated shochu distillery wastewater with *Aspergillus awamori* var. *kawachi*, which was suitable for river discharge after a three-

fold dilution and secondary anaerobic treatment. Aerobic pre-treatment using *Aspergillus awamori* var. *kawachi* reduced the COD from 84.0 to 48.4 g/l and raised the pH from 4.2 to 5.3. There was also an improvement in filterability of the wastewater after fungal treatment. Fitzgibbon *et al*. (1998) compared *Phanerochaete chrysosporium, Geotrichum candidum, Trametes versicolor* and *Mycelia sterilia* at their ability to degrade components of distillery wastewaters. The *Trametes* sp. obtained the greatest colour removal (53 %) in diluted molasses spent wash (12.5 %).

High concentrations of inhibitory compounds found in distillery wastewaters may have to first be removed, or be diluted to allow for biological degradation to take place. Wastewater produced by ethanol production from sugarcane molasses vinasse has a high content of soluble organic matter and intense dark brown colour (Minussi *et al*., 2002). The combination of a high sugar content and polyphenol concentration give these wastewaters a characteristic dark colour. Molasses spent wash can have an extremely high COD (60 to 160 g/l) and a high phenolic content (Driessen *et al*., 1994). Both of these characteristics hamper bioremediation. Generally molasses wastewater samples that have been treated by white-rot fungi have been diluted or pre-treated. An inhibition experiment by Fitzgibbon *et al*. (1998) compared *Geotrichum candidum, Trametes versicolor, Phanerochaete chrysosporium* and *Mycelia sterilia* and found *G. candidum* to be unaffected by gallic acid at the concentrations tested, while the other fungi were inhibited to a varying extent by gallic and vanillic acid. When these fungi were tested in molasses vinasse *Trametes versicolor* reduced the colour by 53 % in a 12.5 % wastewater concentration after ten days of digestion. It was noted that further research would be necessary to exploit bioremediation, as the considerable dilution would negate the use of the treatment system. García García *et al*. (1997) found anaerobic digestion of vinasse to be inhibited by the presence of phenolic compounds. They compared *Aspergillus terreus* and *Geotrichum candidum* for use in the aerobic stage of a dual stage process. Aerobic digestion was primarily for the removal of phenolic compounds, after which the bulk of the COD in the vinasse would be removed using anaerobic degradation. Although *A. terreus* removed 66 % of the total phenolic compounds and 94 % of the *o*-diphenols, *G. candidum* fared marginally better in that it removed 70 and 91 % respectively.

Jiménez *et al*. (2003) studied a dual stage treatment of aerobic and anaerobic degradation of beet molasses alcoholic fermentation wastewater at half strength (COD: 82 g/l). The aerobic degradation potential of three *Penicillium* spp. and *Aspergillus niger* were compared. Of the fungi *P. decumbens* removed the most colour (40 %) while an average of 70 % the phenolic content was removed by all four microorganisms. Chemical oxygen demand removal was similar with maximum removal observed by the fifth day (50.7 to 52.1 %). Later, Jiménez *et al*. (2006) carried out a comparative kinetic study on the anaerobic digestion of untreated vinasse (COD of 80.5 g/l) and vinasse previously fermented with *Penicillium decumbens* (COD of 23 g/l). Both reactors were operated at organic loading rates in the range of 1.5 to 7.5 g COD/(l.day). They found that the combined aerobic/anaerobic process displayed better overall COD removal (96.5 compared with 90.0 %), a decrease of the HRT and greater colour removal. The kinetic constants were found to be higher for pre-treated vinasse than for those of untreated vinasses. This was attributed to the lower levels of phenolic compounds, which resulted in an improved process performance, kinetics and stability. González Benito *et al*. (1997) supplemented distilled beet molasses wastewaters (which had been subjected to anaerobic-aerobic treatment) with sucrose and KH_2PO_4 and obtained 82 % colour removal, 77 % COD removal and 36 % ammonia removal using *Trametes versicolor*.

2.1.7 Products derived from the aerobic treatment of distillery wastewaters

Although the final aim of wastewater treatment is to produce an environmentally benign discharge, a number of biologically derived products may be generated from the treatment process. This can prove advantageous in that, while simultaneously treating a waste, products such as biogas, proteins or polysaccharides may be derived to offset the costs or energy associated with treatment. Anaerobic digestion has gained wider acceptance as greater progress is made governing conditions that allow for more consistent production of biogas, which can be utilised an energy source. Lata *et al*. (2002) estimated that the two polluting industries that had the greatest potential to generate energy from biogas production from their wastes were the pulp and paper industry and distillery industries, at 1131 GWhe/a and 830 GWhe/a respectively. Aerobic treatment can be expensive as initial capital investment for reactors and aeration units often push the price beyond the means of smaller business. A potential way to offset the costs is to derive a product from the waste. Various organic

compounds in the wastewaters may be utilised as a growth source for the production of various types of biomass or to produce valuable compounds from the biomass metabolism such as lipids or proteins or enzymes. Compounds present in the wastewaters may be extracted and be of high value if purified according to the needs of their market. In some instances a lucrative business can result from the utilisation of agro-industrial waste residues.

Water hyacinth (*Eichhornia crassipes*) and channel grass (*Vallisneria spiralis*) have been used for the phytoremediation of lignin and metal-rich pulp and paper mill and highly acidic distillery wastewaters. Wastewater up to a 40 % concentration could be treated and plants were often found to take up metals and toxic compounds. A slurry of the plants that had been used for phytoremediation was digested and produced significantly more biogas than the same plants grown in deionised water (Singhal and Rai, 2003). A cane molasses-based distillery for alcohol production recovered biogas from methanogens using anaerobic fixed film reactors as pre-treatment system. Wastewater was then concentrated through multiple effect evaporators and the concentrate was used for manure or for composting of pressmud (Nandy *et al*., 2002).

Waste activated sludge has been evaluated for its potential to produce poly *b*-hydroxybutyric acid (PHB), a biodegradable plastic. Deproteinised jowar grain-based distillery spent wash and filtered rice grain-based distillery spent wash were used as substrates and yielded 42.3 and 40 % PHB production (w/w) respectively. The addition of $(NH_4)_2HPO_3$ resulted in a 67 % increase in PHB production using raw rice grain-based spent wash (Khardenavis *et al*., in press). Son *et al*. (1996) isolated an *Actinobacillus* sp. EL-9 that synthesised and accumulated PHB in alcohol distillery wastewater during growth. Optimal growth and PHB production were obtained with enzyme-hydrolyzed alcoholic distillery wastewater.

Distillery wastewater has also been utilised as an amendment of substrates used for the cultivation of edible mushrooms. *Pleurotus florida* Eger (EM 1303), *Pleurotus pulmonarius* (Fries) Quelet (EM 1302) and *Pleurotus sajor-caju* (Fries) Singer (EM 1304) were grown on wheat straw (variety UP 2338) and bagasse amended with distillery wastewater that had been treated anaerobically (Pant *et al*., 2006). Better results were obtained using wheat straw with all treatments. *Pleurotus florida* (EM 1303) and *P. pulmonarius* (EM 1302) gave significantly

enhanced yield with increasing levels of wastewater and the greatest biological efficiency was obtained with *P. florida* (EM 1303) at 239 %. Pant and Adholeya (in press) grew selected fungal isolates on wheat straw and corncob comparing water, molasses, potato dextrose broth and distillery wastewater as hydrating agents. The highest laccase synthesis was obtained growing *Aspergillus flavus* TERI DB9 on wheat straw with molasses, while the highest manganese peroxidase activity was found with *Aspergillus niger* TERI DB20 grown on corncob with distillery wastewater. The immobilised *Pleurotus ostreatus* biomass was then used for colour removal of cane molasses based distillery wastewater and obtained a maximum removal of 86 % over a period of 28 days.

The two major costs associated with aerobic wastewater treatment are aeration and disposal of the resultant sludge. The sludge is often rich in single cell protein which may be utilised as a feed supplement. The high content of nucleic acids is not detrimental to animals as uric acid is converted to allantoin, which is readily excreted in urine. The biomass may be heat treated to lower the RNA content to a level suitable for human consumption. As mentioned earlier, Quinn and Marchant (1980) obtained yields of fungal biomass of over 30 g/1 when treating Irish malt whiskey distillery wastewater. Jin *et al*. (2001) obtained yields of 8.5 g/l of fungal biomass protein using *Aspergillus oryzae* and *Rhizopus arrhizus* in an air-lift bioreactor treating starch processing wastewater. Fungal biomass productivity ranged from 0.85 to 0.92 g/(l.h) and the biomass contained 46 to 50 % protein. Later work has shown that grain feed stocks for sheep can be supplemented with the fermented solids that remain after distillation for tequila production (Iñiguez-Covarrubius *et al*., 1996).

Shochu is a popular Japanese alcoholic beverage made from a variety of ingredients including fruit, raw sugar, grains or sweet potatoes and can be distilled once or multiple times. Biological treatment of shochu distillery wastewaters has been investigated in order to produce a useful or valuable product. Barley shochu distillery wastewater was used as a sole nitrogen source for a marine thraustochytrid, *Schizochytrium* sp. strain KH105, to propagate and accumulate valuable lipids including docosahexaenoic acid and astaxanthin (Yamasaki *et al*., 2006). In doing so the soluble COD of the wastewater was reduced by 35 %, the crude protein content by 67 % and total free amino acid content by 85 %. Fungal strains of *Absidia*

atrospora IF09471, *Gongronella butleri* IF08080 and *G. butleri* IF08081 have also been grown in barley-buckwheat shochu distillery wastewater and sweet potato-shochu wastewater supernatants in order to produce chitosan (Yokoi *et al*., 1998). Better chitosan production and a wider pH growth range was observed in the sweet potato-shochu wastewater. *Gongronella butleri* IF08081 produced 730 mg/l of chitosan after five days at pH 5.0 while simultaneously decreasing the COD (49 %), total sugars (51 %), reducing sugars (43 %), protein (45 %), total nitrogen (61 %) and total phosphorus (88 %). Morimura *et al*. (1992; 1994a; 1994b) have used *Aspergillus awamori* var. *kawachi* to treat a distillery wastewater generated from shochu production to produce a saccharifying enzyme, a protease and fungal protein. The feasibility of using thin stillage from a rice-spirit distillery as a substrate for the production of acid protease by *Aspergillus niger* was evaluated by Yang and Lin (1998). Maximum activity of 200 units/ml was achieved in an eight day fermenter culture under the optimal conditions of pH 4 at 30 °C with the addition of 4 % soybean oil with aeration at 1 volume/minute and shaking at 500 rpm. Enzyme production has developed into a significant industry and the costs of generating these valuable enzymes can be reduced by utilising wastes from other industrial sectors as a feedstock, including distillery wastes. Aerobic remediation of distillery wastewaters using white-rot fungi has the potential to produce a fungal enzyme such as laccase.

2.1.8 Introduction to laccase

Laccase is a potential by-product that can be derived from intensive aerobic treatment of distillery wastewaters using white-rot fungi. Laccase was originally discovered in the exudates of the Japanese lacquer tree *Rhus vernicifera* (Yoshida, 1883), and was later demonstrated to be a fungal enzyme as well. More than 60 laccases had been detected from plant, fungal, bacterial and insect sources by 1994 (Thurston, 1994) and more than 100 had been identified from fungal sources alone by 2006 (Baldrian, 2006). Laccase is one of a small group of enzymes called the large blue copper proteins or blue copper oxidases. Ceruplasmin (a mammalian plasma protein) and ascorbate oxidases (plant proteins) are also members of this group. The blue copper oxidases are similar to terminal oxidases involved in aerobic respiration in that they have the ability to oxidise a phenolic substrate while reducing oxygen to water (Thurston, 1994). Like laccases, ascorbate oxidases, cytochrome c oxidase and

ferroxidases have similar copper binding sites that are essential for enzyme activity (Rosconi *et al*., 2005). Natural functions of laccase include lignification and delignification (Hatakka, 1994), formation of humic substances in the soil (Chefetz *et al*., 1998), morphogenesis, and sporulation (Ikegaya *et al*., 1993; Leatham and Stahmann, 1981), growth and development of rhizomorphs (Worrell *et al*., 1986), fungal virulence (Bar-Nun *et al*., 1988) and polymerisation of melanin precursors in differentiated cell walls (Dijkstra and Walker, 1991). Bollag *et al*. (1988) showed the addition of laccase to reverse the inhibitory effects of a number of phenolic compounds upon the growth of *Rhizoctonia praticola* inocula. They attributed the ability of the laccase to detoxify the original phenolic compound by transforming it or by the cross-coupling it with another phenol. Laccase-like enzymes are also involved in the sclerotisation in insects (Chase *et al*., 2000).

Laccase monomers have been found to vary in molecular mass from 50 to 100 kDa, but typical fungal laccases have a molecular mass of 60 to 70 kDa and an acidic isoelectric point around pH 4.0 (Baldrian, 2006). Fungal laccases are generally extracellular glycoproteins and often show considerable heterogeneity in molecular weight after purification, possibly attributable to proteolytic and glycosidic activities in the environment (Wood, 1980). The monomer requires at least four copper atoms for functional catalytic activity. Substrate oxidation by laccase is a one-electron reaction generating a free radical, which is coupled to a four electron reduction of molecular oxygen to water. The initial free radical is typically unstable and may undergo a second enzyme-catalysed oxidation such as the conversion of a phenol to a quinone. It may also undergo non-enzymatic reactions that may result in polymer degradation (due to the cleavage of covalent bonds linking the monomers), ring cleavage of aromatic compounds or even covalent crosslinking of monomer radicals to form dimers, oligomers or polymers. The polymerisation reaction can lead to the formation of an insoluble melanin-like product that may precipitate out of solution. Some of the reactions may lead to the partial demethylation and dehalogenation of aromatic compounds (Claus, 2004). Xu *et al*. (1996b) studied fungal laccases from *Polyporus pinsitus*, *Rhizoctonia solani*, *Myceliophthora thermophila* and *Scytalidium thermophilum* and a bilirubin oxidase from *Myrothecium verrucaria* in order to determine their redox potential, specificity and stability. They observed potentials between 700 and 800 mV (*vs.* normal hydrogen electrode) for the *Polyporus* and

Rhizoctonia spp. laccases, while the other oxidases were lower (approximately 500 mV). Higher redox potentials were found to correlate with higher activity and Xu *et al*. (1996a) speculated that cystine content contributed to stability, while structural differences in the substrate-activation site controlled the redox potential range as well as substrate specificity.

One source of laccase is the group of basidiomycetes known as the white-rot fungi. White-rot fungi are unique among eukaryotic or prokaryotic microbes in possessing powerful oxidative enzyme systems that can degrade lignin to carbon dioxide. These enzymes have a broad substrate specificity and are able to oxidise several environmental pollutants. The vast range of toxic environmental pollutants that are degraded by white-rot fungi also makes these organisms unique and attractive for the bioremediation of polluted sites (Pointing, 2001; Reddy, 1995). White-rot fungi are able to mineralise lignin and use it as a carbon source, whereas brown-rot fungi degrade only the cellulose component of wood and leaf litter. White-rot fungi are able to degrade high strength phenolic wastes due to the activity of lignolytic enzymes such as lignin peroxidase, manganese peroxidase and laccase. Laccase is capable of degrading the phenolic compounds that are normally toxic or recalcitrant to conventional biological waste treatment systems.

It is difficult to define laccase by its reducing substrate due to the wide range of substrates oxidised and their variation from one laccase to the other. This broad substrate range (which includes *ortho* and *para* diphenols, aromatic amines, polyphenols and methoxy substituted phenols), high catalytic constants and its use of molecular oxygen gives it a wide scope of applicability in bioremediation, organic synthesis, immunoassays and biosensors (Yaropolov *et al*., 1994). Laccases are suitable for use in biotechnological applications and have advantages over other enzymes. Laccase catalyses the oxidation of various organic compounds while reducing molecular oxygen to water, without requiring or producing peroxides. Enzymes such as the manganese and lignin peroxidase require peroxide to enable substrate oxidation. Other fungal enzymes, such as alcohol oxidase, produce hydrogen peroxide and the corresponding aldehyde during the oxidation of lower primary alcohols. Molecular oxygen also acts as the electron acceptor (Kerwin and Ruelius, 1969). Laccase has a broader substrate range than the tyrosinases (Saito *et al*., 2003). Additionally, some of the

fungal laccases can also oxidise monophenols such as cresol, and some are even able to oxidise ascorbic acid. However, a limiting factor to the industrial application of laccase is that the optimal condition for catalysis occurs in the acidic pH range and that molecules requiring a redox potential higher than 800 mV can only be oxidised in the presence of small mediatory compounds, which aid electron transfer (Camerero *et al*., 2005).

2.1.9 Laccase synthesis, induction and inhibition

Laccase synthesis is dependent on the strain or genetic manipulation of fungi used, the method used to cultivate it and the constituents of the growth medium. Fungal morphology varies in submerged cultures and is usually classified as either loose mycelia or pellets. Fungal morphology may shift during fermentation between a predominance of pellets or loose mycelia, and very often is a mixture of both types of growth. Loose mycelia lead to highly viscous and pseudoplastic fermentation broths, while pellets are dense, spherical colonies that result in a low viscosity suspension. Mass transfer limitations may occur with both morphology types. The increased viscosity arising from mycelial suspensions can lead to problems in bulk mixing and gas liquid mass transfer, while internal diffusion limitations may occur within pellets. Whether fungi grow as loose mycelia or pellets is determined by a number of factors such as strain, inoculum quantity, age and growth rate, growth medium composition, pH, temperature, mechanical shear forces, oxygen availability, polymer additives and surfactants (Cui *et al*., 1998). It is often difficult to generalise and compare the results from different fungal fermentations as fungi display high sensitivities to slight variations in starting conditions, poor reproducibility and great differences, even within fungal strains (Cui *et al*., 1998). The presence or absence of an inducer, induction time, nature and composition of the culture medium (constituents that provide a nitrogen and carbon source) and type of culture conditions (oxygen availability, pH of the medium, and temperature of cultivation) have a strong influence upon laccase production. The presence of an inducer, its concentration and time of addition are the factors that affect laccase synthesis to the greatest extent. Typical laccase inducers include aromatic and phenolic substances related to lignin or non-aromatic inducers such as ethanol or copper. The effects of an inducer can vary vastly amongst fungal genera. Bollag and Leonowicz (1984) found the aromatic compound 2, 5-xylidine to induce laccase formation amongst *Fomes annosus*, *Pholiota mutabilis*, *Pleurotus*

ostreatus and *Trametes versicolor*, while it had no effect upon laccase production with *Botrytis cinerea* and *Rhizoctonia practicola* and inhibited production in *Podospora anserine*. Copper is an essential component of laccase and its supplementation in the millimolar range has been shown to increase laccase synthesis in a variety of fungi (Hou *et al*., 2004; Chen *et al*., 2003; Galhaup *et al*., 2002a; 2002b; Collins and Dobson, 1997). Laccase synthesis has been shown to be inhibited by high glucose concentrations. Glucose binds to *CreA* sites in *Trametes pubescens* MB 89 and regulates laccase synthesis by repressing *lap 2* transcription (Galhaup *et al*., 2002a).

Various phenolic compounds have been found in wines and those that are heat stable persist in the wastewater after distillation. A number of these and very similar compounds are known inducers of laccase synthesis among various fungal genera. Gallic acid (3, 4, 5-trihydroxybenzoic acid) has been shown to increase laccase production in *Pleurotus pulmonarius* (De Souza *et al*., 2004), *Botrytis cinerea* (Viterbo *et al*., 1993; Gigi *et al*., 1980) and *Trametes pubescens* (Galhaup *et al*., 2002b). Ferulic acid (as well as ethanol) has been used as inducer in *Pycnoporus cinnabarinus* (Lomascolo *et al*., 2003) and *Volvariella volvacea*, while *Coriolus versicolor*, *Pleurotus ostreatus* and *Pholiota mutabilis* were the only three out of sixteen basidiomycetes found by Leonowicz and Trojanowski (1975) to have laccase synthesis induced by ferulic acid. The other hydroxybenzoic acids that have positively affected laccase synthesis include 3, 5-dihydroxybenzoic acid (resorcyclic acid) in *Pleurotus pulmonarius* (De Souza *et al*., 2004) and *Trametes* sp. I-62 (Terrón *et al*., 2004), 2, 4-dihydroxybenzoic acid and *p*-hydroxybenzoic acid in *Fomes annosus* (Haars and Hüttermann, 1983; Haars *et al*., 1981), 4-hydroxybenzoic acid in *Volvariella volvacea* (Chen *et al*., 2003), hydroxybenzoic acid in *Marasmius quercophilus* (Farnet *et al*., 1999) and *p*-coumaric acid (*p*-hydroxycinnamic acid) in *Pleurotus pulmonarius* (De Souza *et al*., 2004). Vanillic acid has been proven to be a good inducer in both *Pleurotus pulmonarius* (De Souza *et al*., 2004) and *Marasmius quercophilus* (Farnet *et al*., 1999). Caffeic acid, (3, 4 dihydroxycinnamic acid) has been shown to increase laccase synthesis in *Pleurotus pulmonarius* (De Souza *et al*., 2004) and *Botrytis cinerea* (Gigi *et al*., 1980), while syringic acid has induced laccase synthesis in *Trametes* sp. I-62 (Terron *et al*., 2004) and *Pleurotus pulmonarius* (De Souza *et al*., 2004).

Substances that inhibit laccase activity are of great consequence to the industrial application of the enzyme. If they are present in sufficient concentrations enzymatic degradation of the substrate will not occur. Consequently the wastewater may require dilutions, which could negate the feasibility of treatment. Laccase inhibition may occur through amino acid residue modification, copper chelation or conformational change of the enzyme (Johannes and Majcherczyk, 2000). Laccase can be inhibited by small anions that bind to the type 2 and type 3 copper and disturb the internal electron transfer. Rosconi *et al*. (2005) observed laccase activity to be fully inhibited by 1 mM NaCN and NaF (electron flow inhibitors). Additionally, laccase activity was inhibited by 100 mM NaCl, which may negate the potential use of this laccase in various industrial processes such as the sugarcane extraction process. *Sinorhizobium meliloti* CE52G laccase was inhibited by Fe^{3+}, Mn^{2+}, and Cu^{2+} ions, sulphydryl organic compounds β-mercaptoethanol and reduced glutathione. Enzyme inhibition by hydroxyl ions can dominate catalysis at a higher pH (Gianfreda *et al*., 1999). Halide inhibition depends on accessibility to the copper atoms and varies between different laccases. Other inhibitors include azide, metal ions (such as mercury cations), fatty acids, sulphydril reagents hydroxyglycine, kojic acid, desferal and cationic quaternary ammonium detergents (Johannes and Majcherczyk, 2000). Cysteine, dithiothreitol, *p*-coumaric acid, kojic acid and thioglycolic acid were shown to inhibit the laccase enzyme reaction using 2, 2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) by Saito *et al*. (2003). Although laccase has an intrinsic sensitivity to halides and alkaline conditions, a polyphenol oxidase from a marine bacterium *Marinomonas mediterranea* remains functional above a neutral pH and displays tolerance towards chloride at 1 M. It has a high specificity for phenolic compounds, particularly methoxyl substituted mono-phenols and catechols an elevated redox potential of 0.9 mV at a neutral pH (Jimenez-Juarez *et al*., 2005).

A number of substances that have been implicated as laccase inhibitors do not necessarily inhibit laccases so much as the substrate or the assay that is performed to assess laccase activity. Johannes and Majcherczyk (2000) tested a number of sulphydryl organic compounds (dithiothreitol, thioglycolic acid, cysteine, diethyldithiocarbamic acid, and sodium azide) as to their effect upon laccase produced by *Trametes versicolor*. They used different test systems utilising ABTS and 2, 6-dimethoxyphenol as enzyme substrates. Only sodium azide was

found to act as a true laccase inhibitor and showed no significant interference with the enzyme tests. All other substances did not significantly inhibit the laccase activity and the previously reported inhibitory effects actually resulted from the reductions of the reaction products such as ABTS cation radical and diquinone or subsequent non-enzymatic interactions during substrate oxidation (complexing with unreacted ABTS resulting in varied spectral characteristics with a concomitant underestimation of enzyme activity).

The addition of some substances such as antichaotropic salts (including $(NH_4)_2SO_4$, K_2SO_4 or Na_2SO_4) have been shown to improve activities of laccase produced by *Sinorhizobium meliloti* CE52G. Dehydration of the hydrophobic regions of the enzyme improves interactions between the hydrophobic substrate and the hydrophobic amino acids near, or at, the active site (Rosconi *et al*., 2005). Substrate and active site hydrophobic interactions can also be enhanced with the addition of sodium dodecyl sulphate, which partly unfolds the enzyme to aid catalysis (Castro-Sowinski *et al*., 2002).

2.1.10 Utilising waste residues for laccase production

Since precedents exist in the literature for the production of laccase from food and beverage wastes, it is logical to extend it to grape processing and winery wastewaters. Generation of any product, even a by-product, carries capital and operation costs which must be outweighed by the benefits of the sale of the product. The market demands for laccase are determined by the extent of its industrial applications. If a waste residue is used to produce the enzyme the product value can offset the treatment process costs.

A variety of agro-industrial waste residues may be utilised as cheap substrates for the synthesis of laccase. Rodríguez Couto *et al*. (2002) examined the potential of barley bran, a common waste from the brewing industry, as a support-substrate for laccase production by *Trametes versicolor* under solid-state conditions. Operating with barley bran at an initial ammonium concentration of 0.2 g/l, laccase activities were enhanced 13-fold in relation to inert support cultures. Laccase production by *T. versicolor* and *T. hirsuta* was improved by supplementing the cultures with 2 mM 2, 5-xylidine and 1 mM copper sulphate. Gómez *et al*. (2005) compared barley bran and chestnut shell waste (from brewing industries and glacé

chestnut waste respectively) as substrates for laccase production using *Coriolopsis rigida*. They found the lignin content to be approximately 60 % in chestnut shells and 21 % in the barley bran. The barley bran substrate resulted in 25 times more laccase than the chestnut shells. Approximately 220 tons of residual plant waste is generated per hectare of banana crop, most of which comprises lignocellulosic-rich leaves and stems. These cellulosic wastes have an enormous potential for exploitation. Although the level of laccase produced did not reach commercial viability, it does offer an alternative to straw, sawdust, wheat bran and bagasse as a substrate for the white-rot fungi (Shah *et al*., 2004). Osma *et al*. (2007b) used banana skins as a support-substrate using *Trametes pubescens* MB 89 and obtained a maximum laccase activity of 1570 units/l. Further work using *T. pubescens* grown on stainless steel sponges in static flask cultures, using mandarin peels as a carbon source and optimised with the addition of soy oil, only obtained laccase activities of 400 units/l (Osma *et al*., 2007a). Kiwi fruit wastes are rich in sugars, proteins, vitamins and minerals. As well as having the physical integrity to serve as a supporting material, their cellulose content stimulates laccase production. Rosales *et al.* (2005) investigated the feasibility of kiwi fruit wastes as a support-substrate for laccase production by *Trametes hirsuta* under solid-state conditions. The highest laccase value was obtained operating at an initial ammonium concentration of 0.15 g/l and with 2.5 g of pre-treated peelings of kiwi fruit. Rodríguez Couto *et al*. (2006) used grape seeds as a support substrate with *T. hirsuta* and found nearly three times as much laccase was produced when compared to an inert nylon sponge support.

2.1.11 Potential industrial applications of laccase and white-rot fungi

Laccase is an important enzyme with numerous potential industrial applications for the enzyme itself, as well as for systems incorporating the fungus that produces the enzyme. Remediation potential has been shown in the food industry for beer production (Yague, *et al*., 2000), olive mill (Jaouani *et al.*, 2005), sugarcane molasses (Bazua *et al*., 1991) and alcohol distillery wastewaters (Fitzgibbon *et al*., 1998). These, and more direct applications of laccase in the food industry, are covered in an excellent review by Minussi *et al*. (2002). Other uses include applications in baking, sugar beet pectin gelation, improvement of food sensory parameters, beer and wine stabilisation (expanded in Minussi *et al*., 2007), fruit juice processing and even as an indicator of *Botrytis cinerea* infection in wine musts. The textile

industry is another major industry where a large amount of research has been conducted to utilise laccase as wastewaters of the dyes are substrates for laccase. Fungal cultures, culture supernatants as well as purified laccase have all shown great potential at colour removal with certain dye-types (Hou *et al*., 2004; Robinson *et al*., 2001; Pointing and Vrijmoed, 2000; Schliephake *et al*., 2000; Swamy and Ramsay, 1999). Natural functions of laccase include lignification and delignification. It is therefore not surprising that laccase and white-rot fungi that produce the enzyme have found applications in the paper and pulp industry. Uses include biopulping (Wolfaardt *et al*., 2004; Archibald *et al*., 1997), biobleaching (Sigoillot *et al*., 2005; Reid *et al*., 1990), improvement of fibre bonding (Felby *et al*., 2002) as well as the treatment of wastewater from paper bleaching (Pedroza *et al*., 2007). Research has also shown laccase to be capable of bleaching dyed denim fabrics (Pazarlioğlu *et al*., 2005), improving the whiteness of cotton in cotton bleaching (Tzanov *et al*., 2003), degradation of hydroxy-polychlorinated biphenyls (Keum and Li, 2004), polyaromatic hydrocarbons (Mayer and Staples, 2002), insecticides (Amitai *et al*., 1998), fungicides (Kang *et al*., 2002) and herbicides (Khadrani *et al*., 1999) and even coal solubilisation (Cohen *et al*., 1990). Laccase is increasingly finding applications in organic synthesis (Karamyshev *et al*., 2003) and biosensors (Shleev *et al*., 2006; Yarapolov *et al*., 2005). Laccase-encoding genes expressed in *S. cerevisiae* have resulted in greater ethanol production in the presence of inhibitory compounds (Larsson *et al*., 2001).

Laccase is one of the few enzymes that has been studied since the 19th century. Research over the past 25 years has shown that there are many potential applications for the laccase enzyme due to its broad substrate range. A number of properties of the enzyme currently prevent its application on an industrial scale. However, some applications that are feasible are hampered by the lack of a cheap, reliable source of the enzyme. The utilisation of a waste residue may allow for much cheaper production of the enzyme.

2.1.12 Conclusions

Several criteria should be considered when selecting a treatment system for wastewaters. These include an eco-friendly process that is flexible enough to handle variable organic and volumetric loads, low capital and operating costs, minimal personal attention and footprint,

while still obtaining the desired degree of degradation without the need for wastewater dilution with potable water. A number of biological systems have been evaluated for wine industry wastewaters, such as anaerobic digesters and activated sludge reactors. Although efficient in COD removal, the presence of certain compounds can reduce treatment efficiency and require long HRTs. Furthermore, the initial capital and running costs of some treatment systems usually put them out of reach of smaller wineries or distilleries.

In the evaluation and reporting of any treatment procedure, sufficient detail must be given to the nature and concentration of wastewater. Distillery wastewaters vary throughout the world, as a reflection of the different raw materials used to produce ethanol. Generally they are high in organic load and low in pH. The presence of phenolic compounds can lead to extremely colour-rich wastewaters and can be toxic to microorganisms. The presence of the inorganic ions may also affect biological treatment. Land application is attractive as a disposal technique but local studies are essential to determine optimum loading rates to rule out the possibility of reductions in crop yields. Evaporation followed by incineration and land disposal is less troublesome but does not produce the energy equivalent of anaerobic digestion. Of the intensive treatment processes, anaerobic treatment has been shown to be satisfactory in many cases although a number of unsuccessful digester trials were attributed to high loading rates. A pre-treatment step to reduce potential inhibitory compounds has the potential to improve the efficiency of anaerobic digestion and enhance biogas production and the digestion kinetics by methanogens. Aerobic treatment has the potential to produce by-products such as fodder yeast, chitosan, enzymes and various lipids.

Laccase has shown remediation potential in wastewaters such as beer production effluent, olive mill wastes, alcohol distillery wastes, dye-containing wastewaters from the textile industry as well as wastewaters from the paper and pulp industry. Laccase may be used to stabilise wines, beers and fruit juices. It has been shown to be capable of remediating soils and waters polluted with chlorinated phenolic compounds, polyaromatic hydrocarbons, nitro-substituted compounds and fungicides, herbicides and insecticides. Additional applications include ethanol production enhancement, coal solubilisation, synthesis of organic molecules, biosensors and the denim and cotton bleaching. From the available literature it is clear that

laccase is an industrially important enzyme and that there are vast applications for the enzyme alone as well as with systems incorporating the white-rot fungi that produce the enzyme. The production of laccase is not only dependent on the strain or genetic manipulation of fungi used, but also the method used to cultivate it. The presence or absence of an inducer, induction time, nature and composition of the culture medium (constituents that provide a nitrogen and carbon source) type of culture conditions (oxygen availability, pH of the medium and temperature of cultivation) have a strong influence upon laccase production. A number of compounds present in wine are known to stimulate enzyme synthesis in various white-rot fungi.

Distillery wastes provide a unique opportunity for fungal laccase synthesis as there are minimal costs associated with media preparation other than pH adjustment and possibly the addition of inducers such as copper and 2, 5-xylidine. Additionally there are numerous phenolic compounds that, although inhibitory to sensitive biological treatment such as methanogen treatment, have the potential to stimulate enzyme synthesis in many genera of white-rot fungi. The use of distillery wastewater as a growth medium has a number of advantages. Energy costs are minimised by the media being heat sterilised in the distillation process. Fungal metabolism and growth decreases the COD and potentially inhibitory phenolic compounds in the wastewater. Under appropriate conditions a high-value enzyme such as laccase can be produced during the treatment process, and mycelial growth may produce a high concentration of fungal protein that may be utilised as a protein source.

2.2 Hypothesis

Pre-treatment of distillery wastewaters using laccase-producing basidiomycetes can lower the COD, colour and concentration of phenolic compounds and increase the pH, thereby rendering the wastewater more amenable to secondary treatment by anaerobic digestion. Simultaneously the phenolic compounds can induce laccase synthesis, thereby producing a high-value product.

2.3 Objectives

The objectives of this study were to remediate wastewaters using fungi and simultaneously produce a high value enzyme as a result of fungal metabolic activities.

The objectives were:

1) Screen selected fungal strains to obtain a species that grows and produces laccase in distillery or winery wastewater.
2) Reduce the total phenolic compounds concentration by the catalytic function of laccase and fungal degradation in the distillery and winery wastewaters.
3) Compare the action of an enzymatic versus fungal treatment system.
4) Reduce the COD of the distillery and winery wastewaters to levels tolerable to anaerobic microorganisms and degrade compounds that may inhibit to anaerobic digestion.
5) Reduce the colour of the distillery and winery wastewaters.
6) Produce laccase at a relatively high concentration of >1000 units/l.
7) Demonstrate that the remediation and laccase production can be scaled up using an airlift, bubble-lift or stirred tank reactor.
8) Demonstrate that laccase production can be enhanced by wastewater supplementation.

Chapter 3

Screening of four white-rot basidiomycetes for the treatment of distillery wastewater and the production of laccase

3.1 Introduction:

Fungal treatment has shown positive results for a variety of wastewaters including those from breweries (Yague *et al*., 2000), olive mills (Jaouani *et al*., 2005; Martirani *et al*., 1996) and vinasse generated from ethanol distillation of fermented sugarcane molasses (Fitzgibbon *et al*., 1995). Fungal growth reduces the chemical oxygen demand (COD) of the wastewater by utilising compounds during growth and metabolic activities. The fungi may secrete valuable enzymes while active in the wastewater. Fungal remediation of a wastewater and synthesis of enzymes are dependent on a number of parameters, including oxygen availability, pH of the medium, temperature of cultivation and presence of constituents in the medium that provide a nitrogen and carbon source. Enhanced fungal laccase synthesis is also dependent on the presence of copper and other inducers, such as aromatic and phenolic substances related to lignin or lignin derivatives. The presence of phenolic compounds extracted from grapes in brandy distillery wastewater could serve to induce the production of high concentrations of the fungal enzyme.

A variety of phenolic compounds present in agricultural waste residues can inhibit the growth of microorganisms, which negatively affects biological treatment processes (Borja *et al*., 1993a). In this chapter a fining agent called polyvinylpolypyrrolidone (PVPP) was assessed to see if there were any benefits to pre-treating the wastewater with a compound that removed a large portion of these suspected inhibitory compounds. Polyvinylpolypyrrolidone acts by adsorption and the amide bonds of PVPP form hydrogen bonds with the hydroxyl groups of polyphenols. It is used in the softening of red wines and preventive and curative treatment of madeirisation of white wines. The use of PVPP can improve a wine's colour, flavour and aroma. Sims *et al*. (1995) showed that PVPP and casein added to white wine before or after fermentation reduced total and flavonoid phenols, lightened the colour, improved resistance to browning and altered the sensory characteristics. Post-fermentation additions of PVPP to red wine also reduced total and polymeric phenols, lightened the colour, reduced the brown colour and significantly altered the sensory characteristics.

There are many fungal species to choose from that have the potential to be used in a biological wastewater treatment process. *Ceriporiopsis subvermispora*, *UD4*, *Pycnoporus*

cinnabarinus and *Trametes pubescens* were selected for the screening experiment, for reasons discussed below.

Niku-Paavola *et al*. (2004) documented laccase production in a strain of *Peniophora*. Not only was this a new feature for the genus, but the laccase displayed good thermostability, with a half-life of approximately 5 hours at 60 ˚C and 10 minutes at 70 ˚C in the culture filtrate. *Peniophora* is a relatively unexplored genus with little earlier literature of biotechnological interest. Ibrahim and Pearce (1980) studied eleven species of white-rot fungi over a 21 day period of solid-state fermentation of barley straw, pea straw, sugar cane bagasse and sunflower hulls. *Peniophora gigantean* displayed the greatest decrease in the lignin content of barley straw and bagasse, the greatest decrease in the cellulose content of pea straw and sunflower hulls and increased the *in vitro* dry matter digestibility of barley straw and bagasse, while *Peniophora cremea* caused the greatest increase in *in vitro* dry matter digestibility in sunflower hulls. An unknown isolate termed *UD4* (possibly a *Peniophora* species) has been shown to produce a remarkably thermostable laccase of great interest to industrial applications (Jordaan *et al*., 2004). The optimum temperature of *UD4* laccase was 70 ˚C and no loss of activity was observed after exposure to 60 ˚C for 9 hours. The laccase also had a high specificity constant, a high substrate affinity and broader substrate range than most isoforms mentioned in available published data, and so was selected for the screening experiments.

The past decade of literature suggested that *Ceriporiopsis subvermispora* was a good candidate for treatment of distillery wastewater. Much of the earlier literature pertaining to *C. subvermispora* was focused on its selectiveness for the lignin component during wood decay (Fukushima and Kirk, 1995). Messner and Srebotnik (1994) observed that a four week pre-treatment of wood chips with *C. subvermispora* prior to pulping had great potential for mechanical as well as chemical pulping on a laboratory scale. Fungal treatment in the presence of wheat bran degraded 75 ± 1 % of the β-O-4 aryl ether linkages in the lignin, which promoted methanogenic fermentation of Japanese cedar wood. Amirta *et al*. (2006) later also reported that *C. subvermispora* was highly efficient at decomposing aryl ether bonds in lignin. Doradoa *et al*. (1999) studied the biological upgrading of wheat straw using

Phanerochaete chrysosporium, *Pleurotus eryngii*, *Phlebia radiata* and *C. subvermispora* over a 60 day solid-state fermentation and observed an increase in straw brightness due to the selective degradation of lignin by *C. subvermispora*. Nagarathnamma *et al*. (1999) found *C. subvermispora* CZ-3 able to decolourise and degrade the first extraction stage wastewater from Kraft pulp bleaching with a relatively low co-substrate concentration. *Ceriporiopsis subvermispora* removed up to 90 % colour (62 % removal without a glucose supplement), 45 % COD, 62 % lignin, 32 % absorbable organic halides and 36 % extractable organic halides in a two day period with a 1 g/l glucose supplement. The pre-treatment rendered the wastewater non-toxic to zebra fish.

Laccase from *Pycnoporus cinnabarinus* has been used in biopulping and biobleaching (Herpoel *et al*., 2002), soil bioremediation (Rama *et al*., 1998), dye decolourisation (Pointing and Vrijmoed, 2000; Schliephake *et al*., 1993) and biopolymer synthesis (Figueroa-Espinoza and Rouau, 1998). *P. coccineus* was used by Jaouani *et al*. (2005) to decolourise olive oil mill wastewaters without an additional carbon source, and as olive oil mill wastes are similar to wine distillery wastes in several of their characteristics, it was probable that *P. cinnabarinus* would demonstrate potential for use in the treatment of wine wastewaters as well. *Pycnoporus cinnabarinus* has been reported to produce laccase as its predominant lignolytic enzyme, with the production of lignin peroxidase and manganese peroxidase not being detected. This is a great advantage for the production of laccase on an industrial scale as it simplifies downstream separation and purification and can also allow for direct use in biotechnological processes (Eggert *et al*., 1996b). Strains have been observed to produce very high activities when cultivated under the correct conditions. Lomascolo *et al*. (2003) observed *P. cinnabarinus* SS3 to produce laccase at a concentration of 267 units/ml in the presence of ethanol at 35 g/l.

Trametes spp. are known to produce high concentrations of laccase. A number of journal articles cite the use of *T. versicolor* for remediation of various wastewaters and soils contaminated with recalcitrant compounds (Chapter 2). Cell extracts from liquid cultures of *T. versicolor* have been shown to solubilise low rank coals (Cohen *et al*., 1990) and the extra-cellular medium containing laccase from *T. versicolor* has been shown to solubilise leonardite

(Pyne *et al.*, 1987). A commercial mixture of horseradish peroxidase and laccase purified from *T. versicolor* was able to dehalogenate polychlorinated biphenyls (Pointing, 2001). Treatment by *T. versicolor* has been shown to increase the brightness and decrease the lignin content of pulp and softwood Kraft pulps (Reid and Paice, 1994; Reid *et al.*, 1990; Paice *et al.*, 1989). *Trametes pubescens* MB 89, the strain used in this study, has been observed to be an exceptional laccase producer with the addition of Cu (II) in the millimolar range to a simple, glucose-based culture medium. Batch cultivation yielded a laccase activity of approximately 330 units/ml (Galhaup *et al.*, 2002b).

3.2 Objectives

The aim of this chapter was to establish whether the four white-rot fungi selected would be able to grow in a brandy distillery wastewater and decrease its colour, concentrations of total phenolic compounds and COD, while producing laccase. *Trametes pubescens*, *Ceriporiopsis subvermispora*, *UD4* and *Pycnoporus cinnabarinus* are all known extra-cellular laccase secretors. These fungi were chosen due to their previously reported ability to produce high concentrations of laccase (*Trametes pubescens* MB 89), a thermostable laccase (*UD4*) or degradation potential (*Ceriporiopsis subvermispora* and *Pycnoporus cinnabarinus*). The reduction in total phenolic compounds could potentially reduce the toxicity of the wastewater to further biological treatment using anaerobic digestion, which was to be assessed using methanogens later in this study. The main objective of this chapter was to modify the wastewater minimally in order to achieve fungal remediation, while simultaneously producing high concentrations of laccase.

3.3 Materials and Methods

3.3.1 Cultures and maintenance

Trametes pubescens MB 89 and *Ceriporiopsis subvermispora* were purchased from Centraalbureau voor Schimmelcultures (The Netherlands, cultures 696.94 and 347.63, respectively). An unidentified wild isolate termed *UD4* was kindly donated by Dr J. Jordaan (Jordaan *et al.*, 2004) and a wild isolate of *Pycnoporus cinnabarinus* was kindly donated by Miss N. Khan (Khan, 2005). All four specimens were routinely subcultured every six to eight months on bacteriological agar (12 g/l, Biolab, Merck Chemicals (Pty) Ltd, Johannesburg)

plates containing 2 % malt extract (Biolab, Merck), 1 % glucose (Saarchem, uniLAB, Merck) and 0.2 % yeast extract (Biolab, Merck) at 28 °C until the first signs of radial growth, then stored at 4 °C until required for inoculation. Agar plugs of the fungus were used to inoculate 100 ml of autoclaved growth medium containing 2 % malt extract, 2 % glucose and 0.2 % yeast extract (all as above) at pH 5.0 which was cultivated at 150 rpm on a bench top shaker (Labcon SP015+UPF75, Maraisburg) at 28 °C. Once the biomass was growing it was again transferred to fresh growth medium and then used to inoculate experiment flasks.

3.3.2 Screening for bioremediation and laccase synthesis

Wastewater was obtained from a brandy distillery near Worcester in the Western Cape Province of South Africa (pH 3.9, 540 mg/l total phenolic compounds and 25500 mg/l COD) and stored at 4 °C. A portion of wastewater was treated by adding PVPP (Sigma Aldrich Ltd, Johannesburg) at 5 % (w/v), shaking thoroughly and allowing to stand for 10 minutes at room temperature (21 °C). Suspended solids were removed by centrifugation twice at 14300 *g* for 15 minutes in a J-10 Beckman centrifuge, followed by filtration through Whatman no. 1 filter paper. The pH of both PVPP-treated and raw wastewater was adjusted to 5.3 using Na_2CO_3 powder (Saarchem, uniLAB, Merck), as two prior experiments (one at pH 3.9 and one at pH 4.5) had yielded no growth of any of the four fungi tested (results not shown). Aliquots of 60 ml were placed in 250 ml Schott bottles, covered with aluminium foil (to prevent contamination) and sterilised by autoclaving for 15 minutes. Triplicate samples of both PVPP-treated and raw wastewater were inoculated with biomass of *Ceriporiopsis subvermispora* (1.38 ± 0.24 g/l), *UD4* (0.89 ± 0.16 g/l), *Pycnoporus cinnabarinus* (1.12 ± 0.39 g/l) or *Trametes pubescens* MB 89 (1.35 ± 0.31 g/l) from the liquid cultures described above. The wastewater samples were shaken at 150 rpm on a bench top shaker (Labcon SP015+UPF75, Maraisburg) at 28 °C for 14 days. Control inocula in distilled water were conducted in duplicate.

The laccase activities, total phenol and COD concentrations and colour absorbance at 500 nm were monitored over a fourteen day period. Chemical oxygen demand samples were measured as a combination of equal portions from each of the three samples for each fungus and wastewater type. Colour absorbance was also compared by combining the three samples,

adjusting to the original pH and comparing 300 µl to the initial wastewater in a multi-wavelength, multi-well plate reader (PowerWave$_x$, Bio-Tek Instruments Inc, Winooska, VT, USA). At the end of the experiment the contents of each flask were centrifuged in a Beckman J-14 centrifuge at 22095 *g* for ten minutes. Pellets were washed with distilled water and oven-dried overnight at 105 °C to determine the dry mass of the fungi (APHA *et al.*, 1998).

3.3.3 Anaerobic digestion of fungally treated wastewaters

The supernatants of the three replicates obtained from final centrifugation for a particular wastewater treated by each of the four fungi were combined. The pH of the supernatant was adjusted to 7.5 using Na_2CO_3 powder (Merck) and 100 ml were inoculated with 5 ml (wet mass volume) anaerobic sludge granules obtained from an anaerobic digester of winery wastewater in the Western Cape. The 100 ml Erlenmeyer flasks were sealed with rubber bungs containing a hypodermic needle that allowed for gas escape. The flasks were placed on the bench top shaker at 25 rpm at 36 °C and digested for fourteen days. Samples were removed on days 1, 3, 5, 7, 10 and 14 and analysed for COD removal and pH change.

3.3.4 Analytical methods

The COD concentration was measured using a colorimetric Spectroquant® method (reagents 14538 and 14539 (Merck), method number 14541, analogous to Standard Method 5220D (APHA *et al.*, 1998). Laccase activity was measured using the PowerWave$_x$ plate reader with the oxidation of 0.1 mM 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate (6)] (diammonium salt, Roche Diagnostics GmbH, Mannheim, Germany) according to Jordaan *et al*. (2004). The total phenolic compounds concentration was determined using Folin-Ciocalteu's reagent (UN3624, Merck) (Khan, 2005). The standard curve was obtained using pure phenol (Saarchem, univAR, Merck).

3.4 Results and Discussion

3.4.1 Screening for bioremediation and laccase synthesis

Pre-treatment with PVPP was tested in order to compare the effects of a lower total phenolic compound concentration upon fungal treatment to that of full strength wastewater. The PVPP treatment of the wastewater decreased the COD by 10 % (from 25.5 to 22.9 g/l), total

phenolic compounds from 540 to 223 mg/l (59 %) and the colour by 46 %. The pre-treatment with PVPP reduced the total phenolic compounds by 59 %, but this accounted for only 12 % of the COD removal by the PVPP treatment.

3.4.1.1 COD removal

Total COD removal efficiencies are shown in Table 3.1. *Trametes pubescens* was the most efficient of the four fungi at COD removal in both the raw and the PVPP-treated wastewater (60 and 61 % respectively). *Pycnoporus cinnabarinus* managed to degrade 50 % of the COD in the raw wastewater and 51 % in the PVPP treated wastewater. *Ceriporiopsis subvermispora* lowered the COD in the raw wastewater by 54 %. All the other results were less than a 50 % decrease in COD. No significant advantage was observed with the use of PVPP and its use detrimentally affected COD removal efficiencies of both *C. subvermispora* and *UD4*. The COD removal efficiencies in this study were lower than those achieved by Quinn and Marchant (1980) using *Geotrichum candidum* in Irish malt whiskey distillery wastewater. They obtained a maximum COD removal of 81 % in an aerated, column fermenter at a temperature of 22 °C. However, this was a trial screening experiment and COD removal would be increased by further optimisation of growth conditions.

Table 3.1: Chemical oxygen demand removal efficiencies for the four fungi in raw and PVPP-treated wastewater (results are from a single reading generated from the pooling of three separate samples).

	***T. pubescens* MB 89**	***C. subvermispora***	***P. cinnabarinus***	***UD4***
Raw wastewater	60 %	54 %	50 %	41 %
PVPP-treated wastewater	61 %	40 %	51 %	32 %

The time taken for the inocula to adjust to their new environment was demonstrated by the lag phase before removal of the COD. *Ceriporiopsis subvermispora* displayed the longest lag phase of the four fungi tested (five days) in PVPP-treated wastewater and raw wastewater, while *Pycnoporus cinnabarinus* was the quickest to acclimatise to the wastewater (Figure 3.1). *Trametes pubescens* and *UD4* both displayed similar acclimatisation patterns, with reductions in COD evident three days after inoculation. The biomass used for inocula for all four white-rot fungi was cultured in a medium containing no wastewater and the lag phase resulted from the inocula's acute acclimatisation. Pre-acclimatising the inocula by exposure to

a low concentration of wastewater in the medium used to culture biomass could possibly have decreased the length of the lag phase.

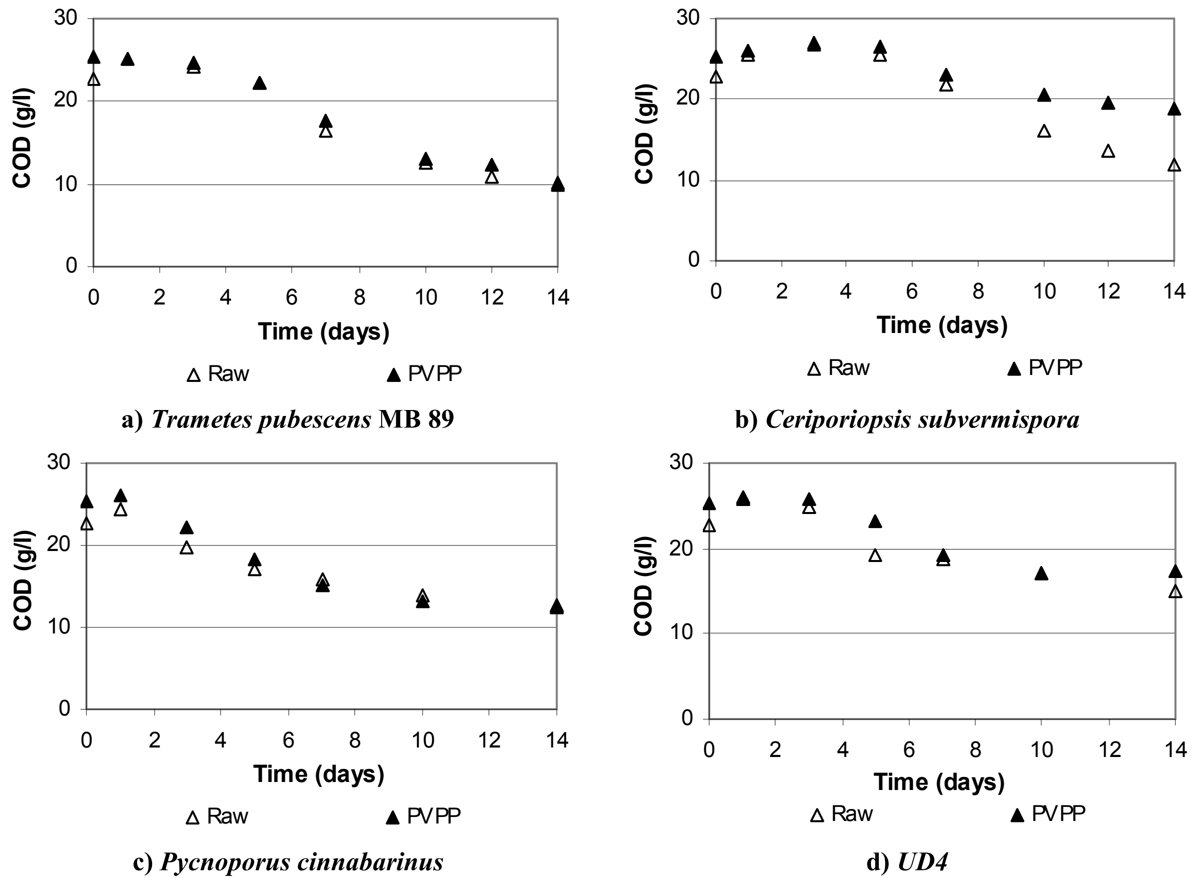


Figure 3.1: Change in COD for the raw (□) and PVPP-treated (Δ) wastewaters inoculated with a) *Trametes pubescens*, b) *Ceriporiopsis subvermispora*, c) *Pycnoporus cinnabarinus* and d) *UD4* (results are from a single reading generated from the pooling of three separate samples).

3.4.1.2 Removal of phenolic compounds

The removal of phenolic compounds from the wastewater by the four fungi is shown in Figure 3.2. *Trametes pubescens* was the most efficient at lowering the total phenolic compounds concentration. It degraded 61 % of the total phenolic compounds overnight, 81 % by day seven and 83 % by the end of the experiment (Figure 3.2a). With the PVPP treatment the total reduction in phenolic compounds was 91 %, which was the highest removal efficiency achieved under these experimental conditions by any of the four fungi tested in this study. *Pycnoporus cinnabarinus* reduced the total phenolic compounds by 38 % (Figure 3.2c), while *C. subvermispora* halved the concentration of the total phenolic compounds (Figure

3.2b). *UD4* was the least efficient fungus at lowering the total phenolic compounds concentration and only removed 28 % of the raw wastewater COD (Table 3.2).

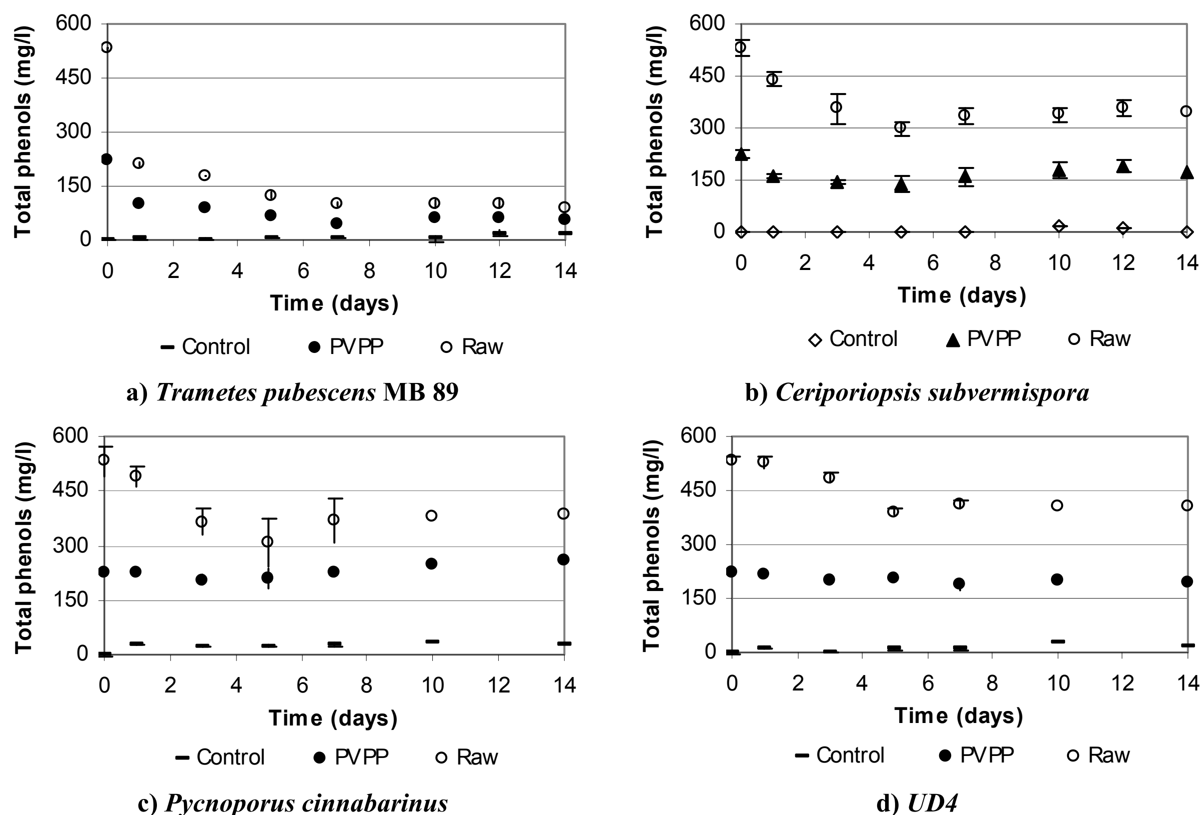


Figure 3.2: Change in total phenolic compounds for the liquid culture inocula of *Trametes pubescens, Ceriporiopsis subvermispora*, *Pycnoporus cinnabarinus* and *UD4* and in raw (Δ) and PVPP-treated (□) wastewater and in the control (◊). (n = 3)

Table 3.2: Highest total removal efficiencies of phenolic compounds obtained in raw and PVPP-treated wastewater. Values represent means ± standard deviation (n = 3).

	T. pubescens	*C. subvermispora*	*P. cinnabarinus*	*UD4*
Raw wastewater	83 ± 3 %	45 ± 6 %	42 ± 7 %	28 ± 1 %
PVPP-treated wastewater	91 ± 1 %	74 ± 1 %	62 ± 2 %	66 ± 1 %

The pretreatment step with PVPP removed 59 % of the total phenols. The decreases in total phenolic compounds after treatment with *UD4* and *P. cinnabarinus* were marginal, and could probably be attributed to adsorption of phenols to the biomass rather than to enzymatic degradation. The concentration of total phenolic compounds in the *P. cinnabarinus* and *C. subvermispora* treated flasks decreased initially, but then increased significantly (Figure 3.2),

such that the concentration of phenolic compounds in the PVPP-treated wastewater containing *C. subvermispora* was greater at the end of the fungal treatment.

The removal of the phenolic compounds by PVPP reduced the ability of *C. subvermispora*, *P. cinnabarinus* and *UD4* to lower the concentration of total phenolic compounds. It may have removed phenolic compounds that acted as mediators or that reacted further with more stable compounds, thereby facilitating overall phenolic compound removal efficiency. Steric hindrance or compound stability could have prevented reactivity. Phenol has been shown to be oxidised by laccase (Kurniawati and Nicell, 2007). When pure phenol was added to a synthetic wastewater made from dilute red wine there was a significantly greater decrease in phenolic compounds after inoculation with *T. pubescens* (Appendix A.1), which indicated that the smaller molecules were serving as mediators or were able to enter less accessible active sites and degrade or precipitate the relatively inert fraction of phenolic compounds.

All of the wastewater samples displayed a decrease in concentrations of phenolic compounds (Figure 3.2). The lowest concentrations were generally measured on the fifth day after inoculation, after which there was an increase (e.g. Figure 3.2b). The most likely reason for the increase in total phenolic compounds was that fungi were secreting or releasing a phenolic compound into the medium. This was substantiated by the increase in total phenolic compounds concentrations of up to 31 mg/l in the *UD4* and *P. cinnabarinus* controls and up to 17 and 23 mg/l in the *C. subvermispora* and *T. pubescens* controls, respectively. These increases in total phenolic compounds occurred in environments that were originally phenolic-free, and corroborate previous authors such as Eggert *et al*. (1996a), who identified a tryptophan-derived metabolite (3-hydroxyanthranilate) that was secreted by *P. cinnabarinus* as a mediator that allowed laccase to oxidise substances with higher oxidation potentials. In this study compounds secreted by these fungi due to the onset of the idiophase resulted in the observed increases in the concentrations of phenolic compounds. Alternatively, phenolic compounds adsorbed to the surface of the inocula subsequently desorbed with time or changing medium conditions, or were released with cellular autolysis. With the onset of the idiophase there was an increase in pH, which could have altered the cell charge such that the net negative charge may have repelled less well adsorbed phenols. However, work carried out

with anion exchange resins led to the conclusion that the phenolic compounds bound extremely well to surfaces and was largely irreversible, as regeneration of the resins led to little removal of the phenols, even after testing desorption in 1 M sodium hydroxide and 1 M hydrochloric acid (Appendix A.2).

3.4.1.3 Colour removal

Table 3.3 compares the change in colour of the differently treated wastewaters as a function of their light absorbance at 500 nm. In the raw wastewater *T. pubescens* obtained the greatest reduction in colour (84 %). *Ceriporiopsis subvermispora* and *P. cinnabarinus* managed to reduce the colour by 54 % and 35 % respectively, while there was no significant change for *UD4*-treated wastewater. With the PVPP-treated wastewater *T. pubescens* obtained the greatest reduction in colour (87 % total reduction). *UD4* reduced the colour by 39 %, while *C. subvermispora* displayed no change in the colour reduction. *Pycnoporus cinnabarinus* increased the absorbance by 17 %. Fitzgibbon *et al*. (1998) tested the ability of *Geotrichum candidum, Trametes versicolor, Phanerochaete chrysosporium* and *Mycelia sterilia* at decolourising molasses spent wash and also found the *Trametes* sp. to be superior to other fungi tested. Moreover, they reported that *Trametes versicolor* decreased the colour (measured as absorbance at 475 nm) by 53 % after ten days. The colour decrease in this study was even more impressive than that attained by Fitzgibbon *et al*. (1998) as the majority of the decrease was observed within the first five days.

Table 3.3: Colour removal efficiencies (%) obtained in raw and PVPP-treated wastewater (results are from the pooling of three separate samples).

	T. pubescens	*C. subvermispora*	*P. cinnabarinus*	*UD4*
Raw wastewater	84	35	54	2
PVPP-treated wastewater	87	-2	-17	39

In this study the colour of some samples and the fungal control samples was shown to increase with PVPP and fungal treatment. This was probably due to the conversion of phenolic compounds to more colour-absorbing compounds such as quinones or dimers, which may have been catalysed by fungal enzymes. Alternatively, an increase in pH may have oxidised some of the less stable phenolic compounds.

The visual results for colour change are shown in the photographs in Figures 3.3 and 3.4. *Trametes pubescens* altered the colour of the raw wastewater from dark brown / black to a yellow / orange colour. Little change is evident in the photos after the treatment of raw wastewater by the three other white-rot fungi. The fungal treatment of PVPP-treated wastewater displayed a slight improvement using *Ceriporiopsis subvermispora* and *UD4*, but *P. cinnabarinus* increased the colour significantly. The greatest colour removal was again obtained with *T. pubescens*, which had turned the PVPP-treated wastewater from brown to a translucent yellow during treatment. All the fungal control flasks were visibly more colour-rich than the original distilled water that had been inoculated, indicating the release of colour-containing compounds by the inocula. *Pycnoporus cinnabarinus* was visibly darker than any of the other controls, indicating that it had secreted or released the most colour-rich compounds over the treatment period, corroborating the assumption in the previous section.

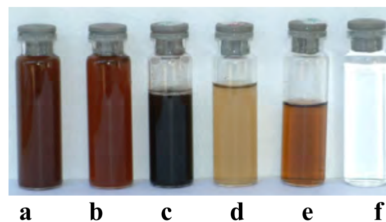


Figure 3.3: Raw WW, b: Raw WW after centrifugation and filtration, c: Raw WW after centrifugation, filtration, pH adjustment to 5.3 and autoclaving, d: PVPP-treated WW, e: PVPP-treated WW after pH adjustment to 5.3 and autoclaving and f: distilled water (control).

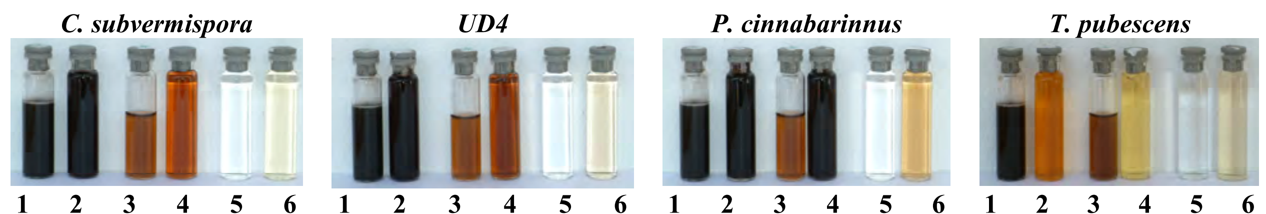


Figure 3.4: Wastewater colour before and after PVPP and fungal treatment. 1: Raw wastewater, 2: Raw WW after fungal treatment, 3: PVPP-treated wastewater, 4: PVPP-treated WW after fungal treatment, 5: distilled water and 6: controls in distilled water.

These results are similar to data reported by Swamy and Ramsay (1999), who tested the ability of *Bjerkandera sp*. BOS55, *Phanerochaete chrysosporium*, *Trametes trogii*, *Pleurotus ostreatus* and *Trametes versicolor* to decolourise the dyes Amaranth, Remazol Black B, Remazol Orange, Remazol Brilliant Blue, Reactive Blue and Tropaeolin O. Pellets of *T. versicolor* were capable of decolourising most dyes and rapidly decolourised repeated additions of the different dyes and dye mixtures without any visually detectable sorption of

any dye to the pellets. Conversely, colour adsorption was evident in this study, as the biomass removed from the wastewater samples was red compared to the cream colour of the controls. However, only a small portion of the phenolic compounds responsible for colour were removed by adsorption. In the next chapter it is shown that the colour actually increased the day after fungal inoculation. Additionally, the haziness that was caused by the small, dark phenolic precipitates that was evident within a day of inoculation disappeared after the fourth day in the *T. pubescens* cultures, probably through adsorption onto or entrapment within the growing fungal mycelia of the pellets.

3.4.1.4 Fungal growth

The pH of the raw and PVPP-treated wastewater samples was adjusted to 5.3 using sodium carbonate, as no growth had been obtained with any of the four fungi during preliminary testing at the original raw wastewater pH of 3.9, nor at a slightly elevated pH of 4.5. At pH 5.3 the dry mass of biomass of all four fungi in the wastewaters increased by 15 to 20 times of that of the original inocula (Table 3.4). The final dry mass values of the four fungal species varied between 1.5 and 4.0 g/l. *Trametes pubescens* was the highest consistent producer of biomass (between 2.9 and 3.4 g/l). *Pycnoporus cinnabarinus* produced 4.0 g/l in one sample, but the biomass production varied more widely, from 2.1 to 4.0 g/l. However, it is difficult to draw meaningful inferences from these values, as they were the dry mass measurements after fourteen days of growth. The fungal masses would probably have been greater at some point prior to this measurement. Weight loss could be attributed to the depletion of internal reserves to maintain metabolism during idiophase as well as the loss of internal compounds and structures that would have been released during the autolysis. *UD4* and *C. subvermispora* produced the lowest concentrations of biomass in the wastewaters. The values of dry fungal mass were low when compared to those obtained by Quinn and Marchant (1980), who obtained yields of fungal biomass from 17.6 to 27.2 g/l using *G. candidum* in Irish malt whiskey distillery wastewater. It was possible that components in (or missing from) the brandy distillery wastewater may have inhibited fungal growth, due to the differences between the feedstocks, or simply that *G. candidum* was a faster-growing and thereby better biomass producing fungus. A similar study by Fitzgibbon *et al*. (1998) also observed fungal growth to be inhibited to a varying extent by the presence of gallic and vanillic acid. *Geotrichum candidum* and *Phanerochaete chrysosporium* growth rates increased in the

presence of increasing concentrations of molasses spent wash (up to 50 % v/v), while the growth of *Mycelia sterilia* and *T. versicolor* was inhibited at spent wash concentrations above 5 % (v/v), illustrating how different fungal genera display different tolerance levels to various components in distillery wastewaters. However, when the mass increase observed in the present study is considered relative to the starting inocula, the fungal mass increased by 16 to 20 times, so Table 3.4 does illustrate that fungal biomass increased substantially during the wastewater remediation. Improved culture conditions such as better oxygenation and strategic cell removal prior to autolysis and depletion of internal reserves could be employed to increase the biomass weight production in the wastewater further, should it be of value as a feed supplement. The fungal biomass could be used to enhance grains or animal feed by serving as a source of protein.

Table 3.4: Comparison of mass and mass increase obtained of the four fungi. Values represent means ± standard deviation (n = 3).

	T. pubescens		***C. subvermispora***		***P. cinnabarinus***		***UD4***	
	Mass (g/l)	Fold increase	Mass (g/l)	Fold increase	Mass (g/l)	Fold increase	Mass (g/l)	Fold increase
Control	0.6 ± 0.1	3.5 ± 0.7	0.7 ± 0.1	7.5 ± 0.7	1.0 ± 0.4	8.5 ± 3.5	0.3 ± 0.1	2.5 ± 0.7
Raw*	3.2 ± 0.2	21.0 ± 1.0	1.9 ± 0.2	20.3 ± 2.1	3.1 ± 0.9	27.0 ± 7.5	2.3 ± 0.5	22.7 ± 4.0
PVPP**	3.1 ± 0.3	20.3 ± 1.5	1.6 ± 0.2	17.0 ± 2.6	2.4 ± 0.3	21.3 ± 2.9	1.7 ± 0.0	16.3 ± 0.6

*Raw wastewater
**PVPP-treated wastewater

3.4.1.5 Laccase synthesis

Trametes pubescens was the only white-rot fungus to synthesise high concentrations of laccase in the wastewater under the conditions tested in this experiment (Table 3.5). The activities of laccase in the raw and PVPP-treated wastewater inoculated with *T. pubescens* were two orders of magnitude higher than any activities obtained by the other three fungi. High activities were measured in the *T. pubescens* controls as well as in the raw and PVPP-treated wastewater. Both wastewaters displayed an initial peak in activity on the third day after inoculation with *T. pubescens*, which was consistent with the production of constitutively-produced laccase (Bollag and Leonowicz, 1984). The onset of the idiophase was marked by the concentrations of the growth substrates decreasing below a critical level. Literature generally observes a large increase in laccase synthesis with the onset of the idiophase. In this work, a rapid increase in activity was detected five days after inoculation, as would be expected with the stationary growth phase. This strain is known to produce very

high concentrations of laccase and a variety of the phenolic compounds found in red wine are known for their inducer ability (discussed in Chapter 2), but the extremely high values obtained by Galhaup *et al*. (2002b) were not reproduced here. The activities measured were two orders of magnitude lower than those reported by Galhaup *et al*. (2002b), however they had cultured their fungus under optimised conditions. The activities observed in this study were more similar to values obtained by Ryan *et al*. (2005). This would be expected in a trial testing with no optimisation of culture conditions (such as carbon and nitrogen supplementation and addition of inducer compounds) other than pH.

Table 3.5: Highest laccase activities (units/l) produced in raw and PVPP-treated wastewater. Values represent means ± standard deviation (n = 3).

	T. pubescens	*C. subvermispora*	*P. cinnabarinus*	*UD4*
Control	1798 ± 38	988 ± 468	92 ± 81	1 ± 0
Raw wastewater	2948 ± 90	4 ± 2	29 ± 40	6 ± 1
PVPP-treated wastewater	2935 ± 98	73 ± 20	36 ± 18	3 ± 1

Pycnoporus cinnabarinus and *C. subvermispora* produced low concentrations of laccase (<100 units/l), while *UD4* produced negligible laccase activities (<10 units/l) in both the raw and PVPP-treated wastewater. The highest recorded laccase activities for *P. cinnabarinus* and *C. subvermispora* in the raw and PVPP-treated wastewater samples were all lower than the activities measured in the control samples (Table 3.5). The lack of laccase production by *UD4* could be attributed to its slow growth rate and the shaking culture conditions. *UD4* has been previously reported to secrete low levels of laccase (Jordaan *et al*., 2004). The low levels of laccase synthesis by *P. cinnabarinus* could be attributed to compounds present or absent from the wastewater, the culture conditions, or that this particular wild-type strain was a poor laccase producer.

The control flasks of *C. subvermispora* displayed relatively high laccase activities, peaking at an average of just under 1000 units/l on the seventh day after inoculation. The large positive laccase activity value in the control for *C. subvermispora* and the correspondingly low values in the wastewater indicated that a compound present in the wastewater negatively affected laccase synthesis by this particular fungus. It also indicated that growth nutrients were transferred to the flasks with the inocula. Laccase synthesis by *C. subvermispora* in cultures containing lignocellulosic materials has been shown to be stimulated by supplementation with

glucose and nitrogen sources (Jones *et al*., 2001; Pandey *et al*., 1999). A lack of these compounds in sufficient concentration in the wastewater may have negatively impacted laccase synthesis. Distillery wastewaters are known to generally have low nitrogen and carbon concentrations and glucose or nitrogen supplementation may therefore increase laccase synthesis. Other researchers using *C. subvermispora* have demonstrated that its ability to mineralise ^{14}C-synthetic lignin was slightly elevated in cultures flushed daily with oxygen, but this decreased the laccase production (Rüttimann-Johnson *et al*., 1993). The fungal cultures were grown with shaking at 150 rpm in the present study, as static culture would be feasible should large volumes of wastewater require rapid treatment. Shaking the flasks increased oxygenation and mass transfer, enhancing biodegradation, but may have inhibited laccase synthesis. All further work in this chapter was carried out using *T. pubescens* as a result of the screening data. Only raw wastewater was assessed, as the PVPP treatment did not provide any advantages to offset the additional cost involved in a pre-treatment step.

The level of laccase produced by *T. pubescens* in the PVPP-treated wastewater was not significantly different to that in the raw wastewater. The higher concentration of phenolic compounds in the raw wastewater (530 mg/l as opposed to the 225 mg/l in the PVPP-treated wastewater) had been expected to lead to greater enzyme induction in the raw wastewater. The experiment was repeated with four different inocula of *T. pubescens* that had been cultured under submerged conditions. Two of the inocula were cultured with 10 % wastewater in the growth medium while two inocula were cultured in plain growth medium. The biomass in this experiment was washed twice with distilled water prior to inoculation by centrifuging, decanting the supernatant, and replacing it with sterilised, distilled water and then used to inoculate the wastewater. There was a significant decrease in enzyme production in the PVPP-treated wastewater to <50 % of the laccase activity measured in the raw wastewater samples (Figure 3.5).

Polyvinylpolypyrrolidone removed more than 50 % of the total phenolic compounds and 10 % of the COD. This would have lowered the concentrations of potential inducers and substrates utilisable for growth. The additional washing step also significantly reduced the level of enzyme activity detected in the control samples, indicating that the levels attained in

the initial screening experiment resulted from the transfer of nutrients with inoculation. There was little difference in the final levels of enzyme production attained with the inocula that had been exposed to 10 % wastewater in the biomass culture and those which had not. However, higher levels were attained earlier with the pre-exposed inocula. This could have been due to the inocula having acclimatised to make use of the available substrates in the wastewater, as well as having developed tolerance towards any inhibitory wastewater compounds.

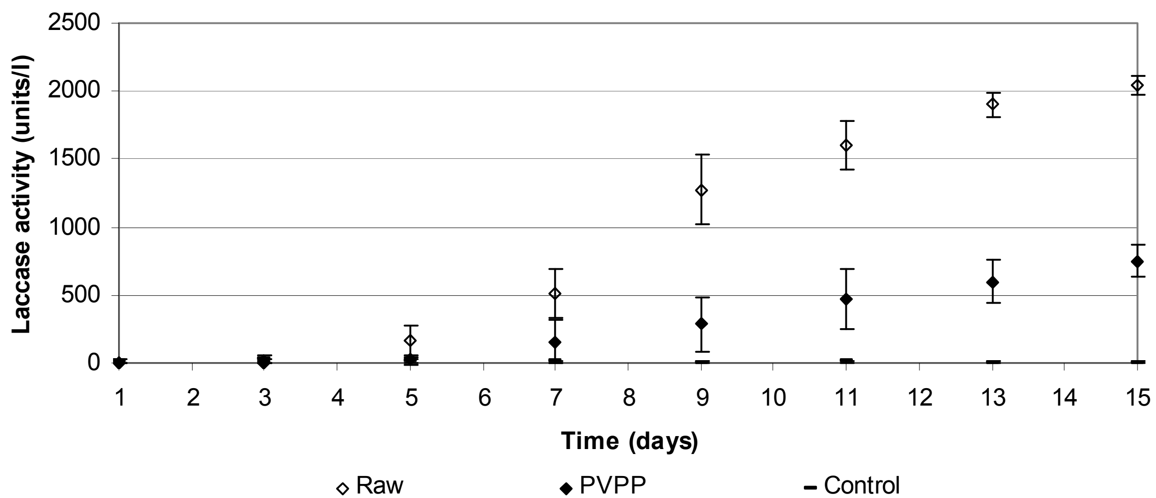


Figure 3.5: Laccase activities for the liquid culture inocula of *Trametes pubescens* in PVPP-treated wastewater (PVPP) (n = 4) and raw wastewater (Raw) (n = 4) from biomass that was exposed to a 10 % concentration of wastewater and no prior wastewater exposure. Error bars indicate standard deviation.

3.4.2 Anaerobic digestion of fungally-treated wastewaters

After fourteen days of anaerobic digestion the fungally-treated samples were all digested to a similar extent (Table 3.6). A total of 92 % to 96 % of the COD was removed by anaerobic digestion after PVPP and fungal treatment. The total COD removal after anaerobic digestion proved very similar for raw and PVPP-treated wastewaters that were treated by the four fungi, indicating that PVPP pre-treatment did not result in any additional benefits for anaerobic digestion subsequent to fungal treatment. No significant differences in the COD removal rates and final efficiencies were observed between the raw and PVPP-treated wastewater control flasks.

If the phenolic compounds in the brandy distillery wastewater were truly inhibitory to the microorganisms, the PVPP-treated wastewater should have displayed increased COD removal with anaerobic digestion. That it did not was contradictory to the results reported by Borja *et*

al. (1993a), who observed that partial removal of some of the organic matter and phenolic compounds from a wine distillery wastewater by aerobic pre-treatment with *Geotrichum candidum* yielded a partially purified wastewater that was more rapidly anaerobically digested than the original wine distillery wastewater for the same COD loading level. It is possible that the decrease in pH counteracted the advantage of a lower phenolic compound concentration in the present study. It was also possible that the phenolic compounds in this wastewater were not inhibitory to the anaerobic digesters at the concentrations present. Just over half the COD was removed by the fungal treatment in fourteen days. The results in Table 3.6 and Figure 3.6 indicate that pH control, a longer hydraulic retention time or a higher sludge concentration would be required to achieve better COD removal by anaerobic digestion alone.

Table 3.6: Comparison of results of COD removal obtained with fungal treatment, anaerobic digestion and a combination of both methods for treatment of PVPP-pre-treated and raw wastewater (Initial COD value: 25.5 g/l COD after PVPP treatment: 22.5 g/l).

	COD removal efficiency (%)			**Wastewater COD concentration (mg/l)**	
	After PVPP treatment	After fungal + PVPP treatment	After anaerobic digestion	After fungal treatment	After anaerobic digestion
UD4 raw*	-	41	93	15100	1773
UD4 PVPP**	24	32	93	17300	1893
P. cinnabarinus raw	-	50	93	12700	1667
P. cinnabarinus PVPP	46	51	93	12400	1853
C. subvermispora raw	-	54	94	13700	1960
C. subvermispora PVPP	33	40	92	17100	1600
T. pubescens raw	-	60	96	11000	1587
T. pubescens PVPP	57	61	94	12300	1120
No fungal treatment raw	-	-	52	-	12167
No fungal treatment PVPP	10	-	53	-	11933

*Raw: raw wastewater
**PVPP: PVPP-treated wastewater

Although the total COD removal by the anaerobic digestion of wastewaters not treated by fungi was considerably lower than that achieved after fungal treatment, the mass of COD removed was as good as the best removal by the fungally-treated wastewaters. The rapid overnight drop in COD (5000 mg/l for the raw wastewater and 3900 mg/l for the PVPP-treated wastewater) for the control samples suggested no limitation by phenolic compounds. It was the rapid drop in pH (shown in Figure 3.6) resulting from the metabolic activity of the anaerobes that hampered COD degradation. It is evident in Figure 3.6 that the pH of the fungally-treated samples recovered after the initial pH drop. The pH values in the control

flasks had dropped to a greater extent and took another eight days before the pH returned to above the metabolically active 6.5+ threshold. However, from days 10 to 14 the COD decreased in excess of 5500 mg/l for the controls and raw and PVPP-treated wastewaters. In a later experiment in Chapter 6 it was shown that controlling the pH led to improved COD removal in anaerobic digestion of wastewaters that were not fungally-pre-treated.

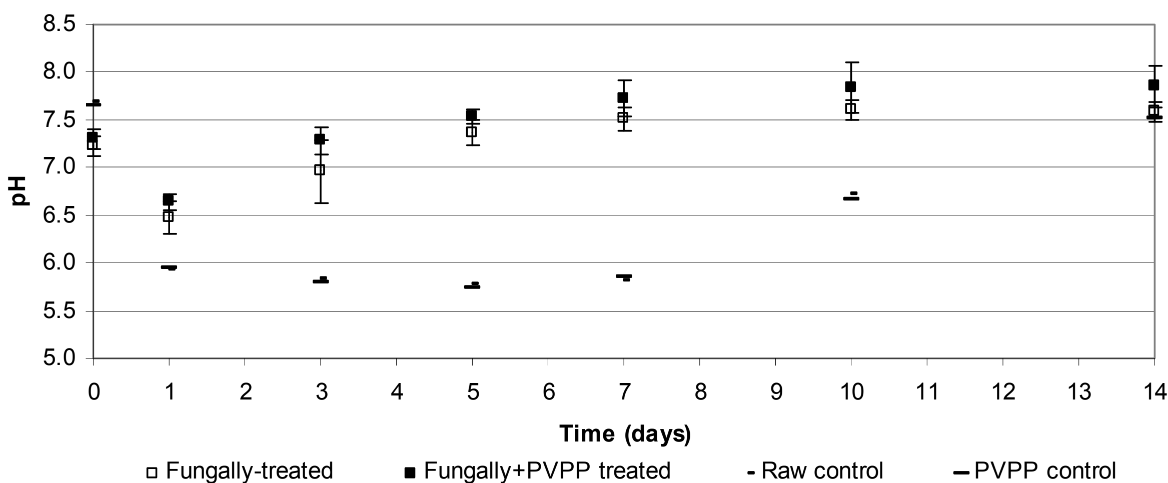


Figure 3.6: Change in pH for the anaerobic digestion of the fungally-treated wastewater (n = 4), fungally and PVPP-treated wastewater (n = 4). Raw wastewater (n = 1) and PVPP-treated wastewater (n = 1) that were not treated by fungi served as controls. Error bars indicate standard deviation.

3.5 Interim summary and conclusions

Pre-treatment with PVPP did not truly enhance fungal wastewater treatment and it decreased laccase synthesis with *T. pubescens* MB 89, which gave the most promising results of the four fungi tested. Its use would possibly be advantageous when testing a wastewater with a higher content of toxic phenolic compounds. Pre-treatment of the wastewater with PVPP had little advantage other than a slight improvement in total phenolic compounds removal with *T. pubescens* MB 89. However, laccase synthesis in PVPP-treated wastewater was significantly lower than with raw wastewater. Further, the cost of the PVPP relative to the small improvement in wastewater quality would preclude its use.

From the data obtained in this experiment it appears that *T. pubescens* had the greatest potential of the four species screened for treatment of the wastewater. *Trametes pubescens* MB 89 displayed the greatest capacity for COD, total phenolic compounds and colour

removal from the raw as well as the PVPP-treated wastewater. It also produced higher laccase concentrations than all other cultures tested.

Pre-treatment with phenolic compound removal using PVPP prior to anaerobic digestion (with no fungal treatment step) did not enhance the COD removal compared to digestion of raw wastewater that had received no prior treatment at all. This indicates that the phenolic compounds present in this wastewater were not as inhibitory to anaerobic digestion as has been reported in literature. The reason for the resilience of the anaerobic sludge used in this experiment was due to it being a granulated sludge that had been obtained from a wine-distillery wastewater fermenter. Not only would the prior exposure to the various phenolic compounds present in the wastewater have resulted in the natural selection of a more resistant microbial community, but granular sludge has been noted in literature to be more resilient than conventional sludge (Keyser *et al*., 2003).

The emergence of *T. pubescens* as both the producer of the greatest amounts of laccase and the best fungus for treatment of the wastewater was a key finding of this chapter, and made it the leading candidate for further study. The following chapter will examine the potential of *T. pubescens* to remediate brandy distillery wastewater in greater detail.

Chapter 4

Bioremediation of a brandy distillery wastewater and the production of laccase using *Trametes pubescens* MB 89 in shake-flask cultures and a 50 l bubble-lift reactor.

4.1 Introduction

Distilleries often produce large volumes of liquid wastes that are high in organic strength and a major source of soil and water pollution (Blonskaja *et al*., 2003). Wine distillery wastewater is acidic, varies in colour from brown to dark red due to the presence of phenolic compounds (anthocyanins in particular) and has a high BOD (5 to 33 g/l), COD (12 to 1112 g/l) and electrical conductivity (0.18 to 1.16 S/m). It also contains high concentrations of phenolic compounds ranging from 65 to 766 mg/l (Bustamante *et al*., 2005), which are inhibitory to biological treatment, as the compounds adversely affect the metabolic activity of some microorganisms (Borja *et al*., 1993a; 1993b). Aerobic pre-treatment of distillery wastewater using white-rot fungi has the potential to decrease the concentration of phenolic compounds, colour and COD.

Flask cultures allow for the simultaneous assessment of multiple parameters and comparison with relative ease. A greater liquid/air interface area can be created by maintaining a high flask volume to sample volume ratio, thereby allowing for easier diffusion of oxygen into a liquid medium. Shake-flask cultures make use of a constant agitation that increases oxygen diffusion into the medium and carbon dioxide diffusion out of the medium. Shaking facilitates mixing of the culture medium and prevents concentration gradients forming around the inoculum. Constant mixing increases the mass transfer of nutrients and increases the rate of fermentation/digestion. With fungal inocula the agitation allows for shear forces and collisions which increase fragmentation of mycelia, thereby generating new points of growth of mycelia. These mycelia can either remain growing as hyphal strands/mycelia or develop into larger, compact spheres of growth known as pellets.

The greatest problem in aerobic submerged culture is maintaining sufficient oxygenation that can support biomass respiration. Oxygenation can be provided by bubble dispersion into the medium, or by stirring the medium such that oxygen can diffuse into the surface layer of the liquid. Bubble-lift reactors offer advantages over mechanically stirred tank reactors for wastewater treatment using aerobic monocultures. Aeration provides oxygen and mixing. There is no requirement for submerged moving parts, which consume energy, have associated

costs, act as surfaces for attachment and are a potential source of contamination through the bearings and seals.

4.2 Objectives

The objectives of this chapter were to investigate the use of a white-rot fungus, *Trametes pubescens* MB 89, to lower the COD and total phenol concentration of a brandy distillery wastewater (thereby reducing the possible toxicity), to increase the pH and to decrease the colour. Concurrent to bioremediation, a secondary objective was to produce laccase at a high concentration. This was assessed in shake-flask cultures, a number of air-lift and bubble-lift reactors and a continuously-stirred tank reactor. The most promising reactor was scaled up to 50 l and assessed for bioremediation efficiency and enzyme production.

4.3 Materials and methods

4.3.1 Culture and maintenance

Trametes pubescens MB 89, purchased from Centraalbureau voor Schimmelcultures, Netherlands (culture 696.94), was routinely subcultured at 28 °C on bacteriological agar (12 g/l, Biolab, Merck Chemicals (Pty) Ltd, Johannesburg) plates containing 2 % malt extract (Biolab, Merck), 1 % glucose (uniLAB, Saarchem, Merck) and 0.2 % yeast extract (Biolab, Merck) and stored at 4 °C.

4.3.2 Flask cultures

The pH of the undiluted, raw wastewater (same wastewater as in section 3.3.2) was adjusted to 5.3 (as this had allowed for excellent growth in Chapter 3) using Na_2CO_3 powder (Saarchem, uniLAB, Merck) and 100 ml aliquots were placed in 500 ml Erlenmeyer flasks, covered with aluminium foil (to prevent contamination) and autoclaved. The flasks were then inoculated with rinsed *T. pubescens* MB 89 biomass (1.27 ± 0.31g/l) from stock cultures that had been exposed to a 10 % concentration of wastewater containing 2 % malt extract, 1 % glucose and 0.2 % yeast extract (all Merck, as in Chapter 3). The flasks were placed in a shaking incubator (Labcon) at 150 rpm at 28 °C for 15 days. Wastewater flasks were conducted in triplicate and control inocula (distilled water) in duplicate. Samples were taken from the flasks daily and centrifuged in 1.5 ml Eppendorf containers at 9660 *g* for two

minutes (Heraeus Biofuge, Germany). The supernatant was aspirated, diluted appropriately and tested for laccase activity, total phenolic compounds concentration, COD and pH using the methods described in Chapter 3 (section 3.3.4), and colour as indicated by a change in absorbance at 500 nm (PowerWave$_x$, Bio-Tek Instruments Inc, Winooska, VT, USA). Absorbance at 500 nm was measured by mixing a 100 µl sample with 200 µl distilled water in 33 mM phosphate buffered saline at pH 7. Values were normalised by conversion to a percentage of the untreated wastewater.

4.3.3 Bubble-lift bioreactor

Trametes pubescens MB 89 was cultured using a number of test methods, including shake-flask cultures, bubble-lift reactors, air-lift reactors, immobilised airlift reactors and in a continuously-stirred tank reactor varying in volume from 1 to 27 l. Bubble-lift reactors yielded the best results so a 50 l bubble lift bioreactor was constructed and assessed (Figure 4.1). A 5 l inoculum was cultured in a 10 % concentration of wastewater containing 2 % malt extract, 1 % glucose and 0.2 % yeast extract and aseptically added to 45 l autoclaved, full strength, raw distillery wastewater. The initial biomass concentration was 0.73 ± 0.08 g/l and the treatment took place at 28 °C. Hydraulic and mean cell retention times were 15.5 days. Aeration was provided by compressed air, bubble-diffusion at the base of the reactor through an inline 0.22 µm filter (Millipore SA, 67120, Molsheim, France) at approximately 60 l/minute. The initial organic volumetric loading rate was 2.72 kg COD/m^3.day^{-1}. Samples of mixed liquor were removed every 12 hours for analysis. Laccase activity, total phenolic compounds concentration, COD concentration and colour were monitored daily according to the methods described in section 4.3.2. Subsamples (25 ml) for dry mass determination were stored at 4 °C and analysed at the end of the experiment using Standard Method 2540 D (APHA *et al*., 1998).

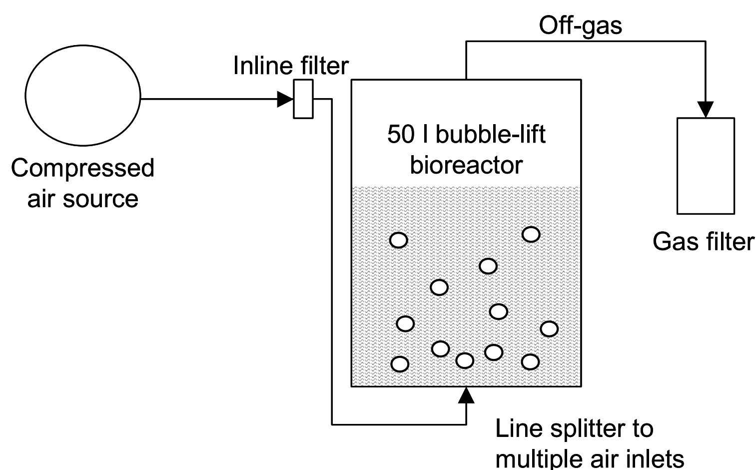


Figure 4.1: Schematic diagram of 50 l bubble-lift bioreactor.

4.4 Results and discussion

4.4.1 Flask cultures

4.4.1.1 Chemical oxygen demand

The wastewater COD decreased from 25.5 to 5.5 g/l. The greatest decrease occurred over the first seven days, and the reduction rate slowed down thereafter. This is a significant COD reduction for a pre-treatment step. The decrease in the COD removal rate coincided with a metabolic transition, as indicated by a simultaneous increase in pH (Figure 4.2) as well as an increase in laccase activity (Figure 4.4). These results were promising when compared to results obtained by Malandra *et al*. (2003). They developed a 250 l prototype rotating biological contactor (RBC) that proved to be highly effective for the treatment of fresh winery wastewater. Wastewater from a cellar had 43 % of its COD removed with an average retention time of one hour (influent COD of between 3 and 9 g/l) over a period of three months. The RBC was subsequently evaluated at a bottling plant and resulted in an average decrease in COD of 34 % and a pH increase of 0.83 units with a retention time of one to four hours. However, the system was completely ineffective for the treatment of distillery wastewater (2 % removal after 3.2 hours retention time) even though thick biofilms had developed on the RBC discs. It was suspected that the microorganisms were inhibited by the high concentration of refractory compounds in the distillery wastewater such as polyphenols or organic acids.

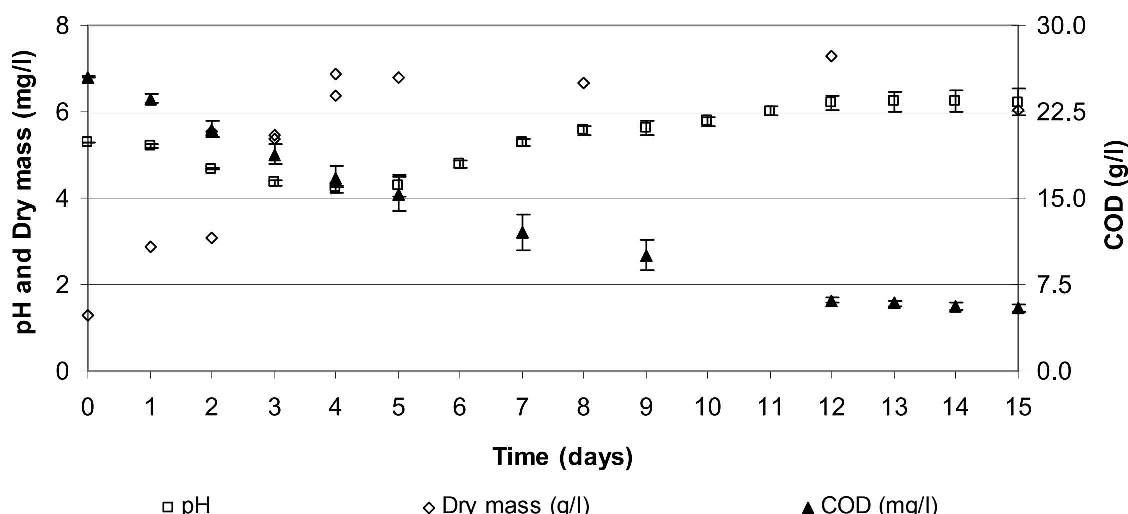


Figure 4.2: Change in pH and COD of wastewater plotted with dry mass during shake-flask treatment of brandy distillery wastewater using *T. pubescens* over a 15 day period. Error bars indicate standard deviation and in some cases are too small to be visible.

4.4.1.2 pH

The pH in the flask cultures (Figure 4.2) decreased over the first four days (attributable to metabolic activities involved in cellular growth) and then increased after the fifth day (attributed to metabolic activities during the stationary growth phase). The result concurs with Galhaup *et al*. (2002b), who reported a pH change from 5.0 to 3.6 during the growth phase and a sharp increase in pH when carbon source in the medium was exhausted. Elevated pH values after fungal treatment (pH 6.5 as opposed to pH 4.0) would better suit a secondary biological treatment system using anaerobic digestion as it would require a nominal adjustment to the desired pH range of 7.0 to 7.5.

4.4.1.3 Total phenolic compounds

The concentration of total phenolic compounds was reduced by 80 ± 4.6 % (Figure 4.3). The majority of the removal occurred within the first two days, with a minor decrease thereafter. The large initial reduction could be attributed to enzymatic action, while the further removal of phenols can be attributed to cellular degradation or possibly synthesis of mediators that further aided enzymatic degradation (Eggert *et al*., 1996a). The removal of phenolic compounds was related to the reduction in colour, as the dark colour of red wines is attributable to a portion of the phenolic compounds present (anthocyanins). The recalcitrance to enzymatic degradation may have been due to structural inaccessibility of the active site of

the colour-containing compounds or an E° higher than that which the enzyme was capable of oxidising (discussed in section 3.4.1.2).

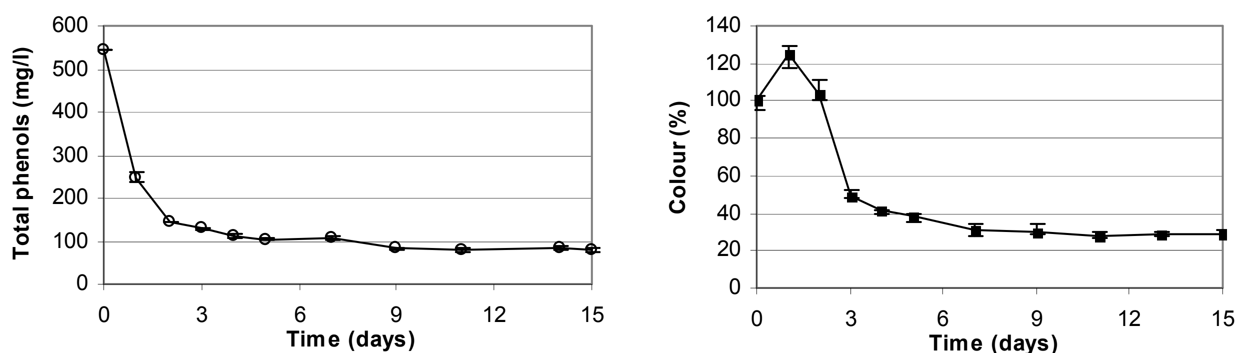


Figure 4.3: Change in total phenolic compounds concentration (left) and colour (right) over a 15 day period.

4.4.1.4 Colour

There was an initial overnight increase in colour (Figure 4.3), simultaneous with the greatest reduction in total phenol levels. The colour increase could be attributed to either the conversion of the phenolic compounds into a darker compound by the action of laccase, or to the formation of small aggregates that were not removed by centrifugation (a fine, dark haze had developed after inoculation). The colour decreased quickly though, indicating that it was either unstable, degraded naturally or was degraded by the biomass. Later work done with a wine lees and crudely purified laccase suggested that the darker compound was not unstable and that fungal growth and metabolism had been responsible for the degradation of the transformed phenolic compound (Chapter 5). The greatest colour reduction occurred between days one and three after inoculation. This was due to enzymatic action, as some laccase substrates are known to polymerise and form aggregates that precipitate out of solution, and primary metabolism that resulted in compound degradation during growth and metabolism (Rodríguez Couto *et al*., 2004). The extra-cellular liquid produced by *T. hirsuta* in submerged cultures has been shown to efficiently decolourise several synthetic dyes *in vitro* with short incubation times. At pH 4.0 Bromophenol Blue and Indigo Carmine were nearly totally degraded after 2 and 5 hours respectively. Nearly 70 % of Methyl Orange degraded in 5 hours, while only 21 % of Poly R-478 was degraded after 7 hours (Rodríguez Couto *et al*., 2004). This demonstrated that the dye decolourisation Rodríguez Couto *et al*. (2004) observed was due to enzymatic degradation and not fungal metabolic degradation. Their study also

showed that some compounds were not easily susceptible to laccase degradation. This would be problematic for enzymatic application to wastewaters such as those generated from wine-related industries, as a multitude of diverse phenolic compounds are responsible for colour. Some compounds may be removed by precipitation, but others may be converted into compounds more colour-rich than the parent compounds.

4.4.1.5 Laccase production in shake-flask cultures

Laccase activity in the flask cultures showed a small initial peak on the third day, associated with fungal growth (Figure 4.4). The activity decreased slightly until day five, increased between days six and twelve and thereafter remained static. This is a normal trend in laccase synthesis and has been observed by other researchers (Collins and Dobson, 1997). Laccase is first produced in lower concentrations constitutively with the biomass increase, then synthesised at a much greater rate during the idiophase. This strain is known to produce high concentrations of laccase and the variety of the phenolic compounds found in red wine (discussed in Chapter 2) may have stimulated laccase synthesis. The biomass increased constantly up until day four, indicating the end of the primary growth period. This was shown two days thereafter with the rapid increase in laccase synthesis, indicating the exhaustion of some factor, such as the carbon source, and the subsequent hypersecretion of the enzyme. By the twelfth day the laccase activity remained static, indicating the commencement of the autolytic stage, as the biomass value was shown to have decreased by day fifteen.

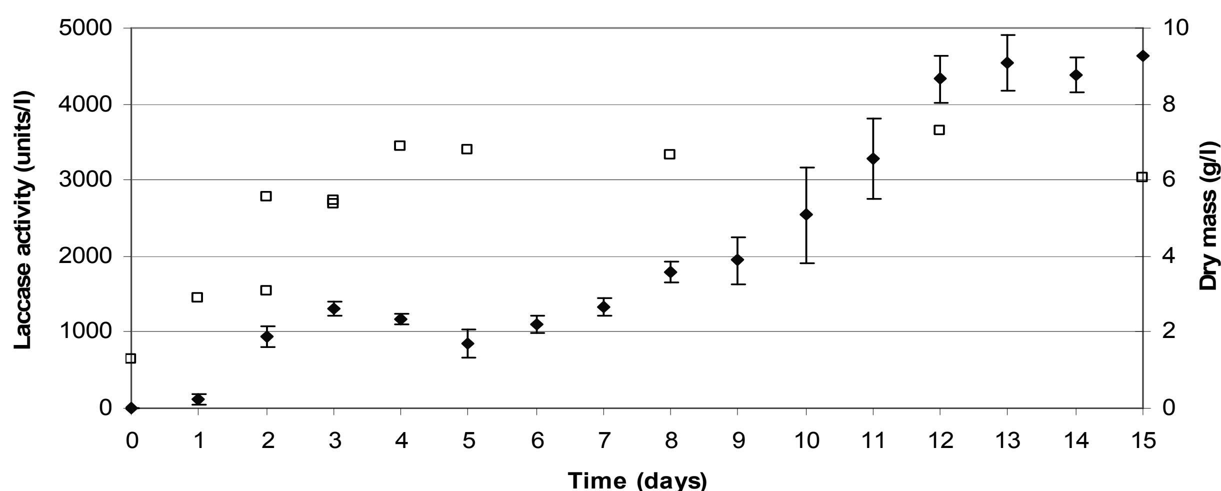


Figure 4.4: Production of laccase (♦) (*n* = 4) and dry mass (□) (*n* = 1) by *T. pubescens* in flask cultures over a 15 day period. Error bars for laccase production indicate standard deviation.

4.4.2 Bubble-lift bioreactor

The 50 l reactor worked well except for the lack of a moisture-trap in the laboratory air supply, which led to moisture collecting in the air filter and detrimentally affecting aeration twice. The first filter blockage led to a pressure build-up that caused the aeration hose to disconnect after the second day of the experiment. The second filter blockage did not cause the aeration hose to disconnect, but did significantly decrease the flow rate on the seventh day of the experiment. The fungal culture was oxygen-starved for up to 12 hours both times and this reflected in the data from all the assays. However, the fungus recovered and produced a high concentration of laccase (12800 units/l) and decreased the COD by 61 % and the total phenolic compounds by 77 % of their original concentrations. The colour had decreased by 70 % by day 10 but then increased during the final five days of the experiment.

Although a large degree of frothing had been anticipated, the foam layer was less than 20 cm in height. The advantage of using two air outlets with a relatively large bore (1 cm inner diameter) instead of multiple smaller outlets (3 mm inner diameter) or sintered plate was that larger bubbles were formed. These larger bubbles prevented large scale frothing that had been seen in other reactors.

4.4.2.1 Wastewater remediation

The COD decreased sharply after inoculation until the first interruption in aeration (Figure 4.5a) and then remained static for two days thereafter, probably recovering from the oxygen starvation. The COD then decreased again until day six, when the aeration was hindered again by a partially blocked air filter. The COD remained static for 24 hours after the filter had been replaced and decreased again over the next four days. There was no further significant change in COD values from days twelve to fifteen, indicating that the majority of the COD that was degradable by the fungi had been utilised or broken down. The results obtained here indicate that COD removal in the bubble-lift reactor was similar to that observed in the flask cultures, where removal after the twelfth day was also very low. The removal rate over the first two days and between days four and six indicated much better removal efficiency than that of the shake-flask cultures. If the rates are plotted without the COD-removal stalls incurred by inefficient aeration, the same total COD removal as observed in the flasks over fifteen days

would have been attained in the bubble-lift reactor before the sixth day, approximately half the time required in the shake-flask cultures.

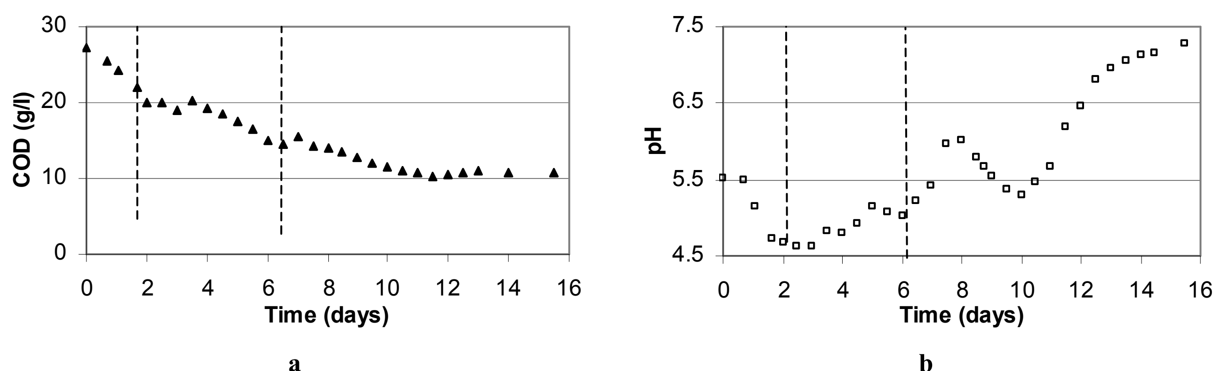


Figure 4.5: Change in a) COD and b) pH of wastewater during a 15.5 day period of fungal treatment. Relative Std dev <5 % (duplicate samples taken at same time). Vertical dotted lines indicate interruptions in aeration.

The two periods of aeration inefficiency resulted in disjointed trends for all the parameters tested. The most rapid and distinct changes were observed with the pH measurements, indicating multiple shifts in metabolism that were the result of oxygen starvation-induced cell death. Although the trends can not be adequately explained without conjecture, the peaks and troughs observed in the pH evolution (Figure 4.5b) related well to the disjointed results for the other parameters. The pH dropped sharply over the first 48 hours, which was normal as literature indicates this is typical with primary metabolism and the consumption of easily utilisable sugars in the medium (Galhaup *et al*., 2002b). After the first oxygen shortage the pH declined very slightly over the next 24 hours, and then increased until day five. In shake-flask cultures the pH had only increased after the fourth day after inoculation and would then increase for the remainder of the experiment. The bubble-lift reactor then displayed another decrease in pH between days five and six. Day six was when the next large oxygen starvation occurred due to a partially blocked air filter. The pH increased rapidly between days six and eight and then displayed a highly unusual decrease until day ten. Thereafter the pH increased rapidly and tapered off after day twelve.

The greatest reduction in total phenolic compounds concentration occurred during the first 48 hour period of the experiment (Figure 4.6a), as it had with the shake-flask cultures. The rapid decrease was again associated with enzymatic activity. There was an abrupt end to phenolics

removal with the loss of aeration. The concentration of phenolic compounds remained static thereafter until half-way through day 5, after which it again decreased until day seven. This indicated that the further removal of phenolic compounds was due to primary metabolism, as it took the fungal inoculum a few days to recover and increase in biomass after the oxygen starvation (Figure 4.7). The decrease in total phenolics coincided with an increase in biomass and the onset of greater laccase synthesis (Figure 4.7) indicating the growth phase shift that would normally be associated with the onset of secondary metabolism. This second decrease in phenolic compounds concentration ended abruptly and coincided with a peak in the pH, potentially indicating another shift in metabolism that no longer resulted in the digestion of phenolic compounds. As the pH decreased, the total phenolic compounds increased slightly, but remained fairly constant from day ten until the end of the experiment, as the pH increased again.

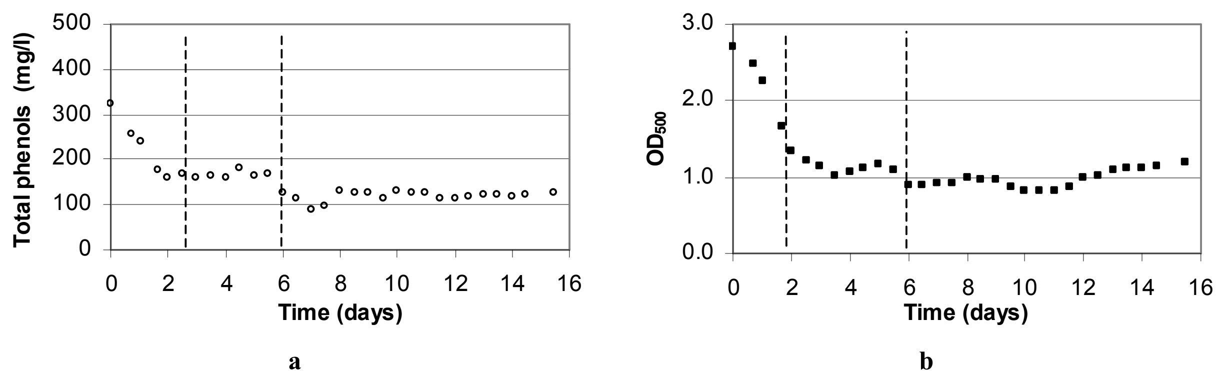


Figure 4.6: Change in a) total phenolic compounds and b) colour of wastewater during a 15.5 day period of fungal treatment. Std dev <5 % (duplicate samples taken at same time). Vertical dotted lines indicate interruptions in aeration.

The colour decreased sharply over the first 48 hours and at a slower rate over the next 36 hours. Thereafter it fluctuated but generally decreased until day eleven, after which it increased for the remainder of the experiment (Figure 4.6b). The removal of colour can be correlated to the decrease in phenolic compounds concentration. Another trend is discernable if the colour change (Figure 4.6b) and pH shifts (Figure 4.5b) are compared. Each of the slight increases in colour coincided with an increase in pH, and the dips in colour related to a dip in pH. From a visual perspective the colour changed from dark brown to a light orange, which coincided with the greatest removal in phenolic compounds. The colour darkened slightly

over the last few days of the experiment, coinciding with greatest pH increase and cell autolysis.

4.4.2.2 Laccase production in the 50 l reactor

The highest laccase activity measured in the 50 l bioreactor was in excess of 12 000 units/l (Figure 4.7). These activities were in accordance with values obtained by Ryan *et al.* (2005) under submerged conditions and very favourable compared to those obtained by Osma *et al.* (2007b). Osma *et al.* used banana skins as a support-substrate using the same *Trametes pubescens* MB 89 strain from Centraalbureau voor Schimmelcultures and obtained a highest laccase activity of 1570 units/l. Conversely, the results were an order of a magnitude lower than those reported by Galhaup *et al.* (2002b). This could have been attributed to the inducers and glucose concentrations cited by Galhaup *et al.* not being used in this set of experiments. Nonetheless, high laccase activities were attained using wastewater as a growth medium, while simultaneously treating the wastewater.

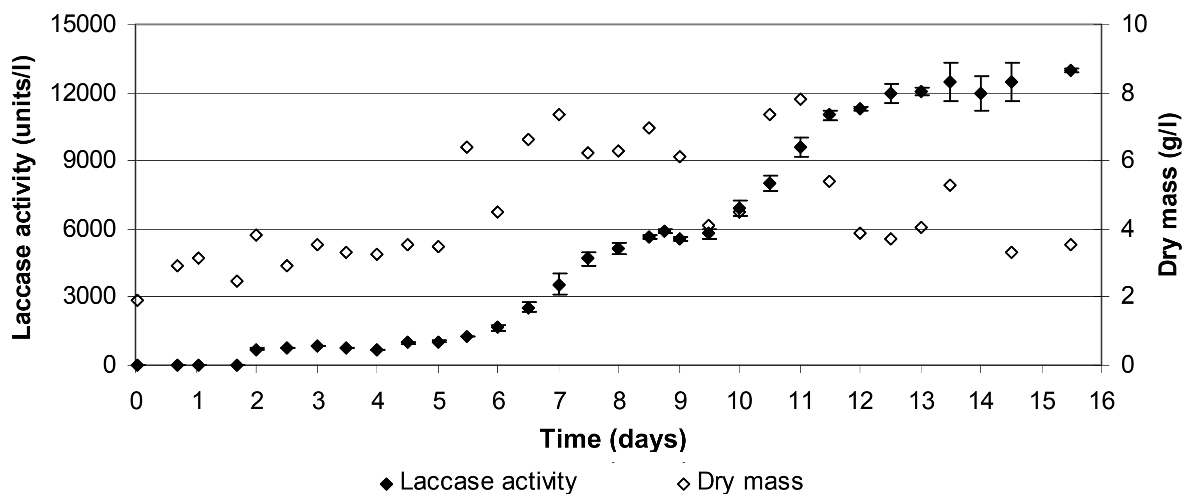


Figure 4.7: Production of laccase (♦) and dry mass (□) by *T. pubescens* in the 50 l bubble-lift reactor. Error bars indicate standard deviation.

4.5 Interim summary and conclusions

Trametes pubescens MB 89 treatment was shown to be effective as a biological wastewater treatment step, attaining 80 ± 4.6 % total phenol removal, 71 ± 1.6 % colour removal, 79 ± 1.1 % COD removal and pH increase from 5.3 to near-neutral. The values attained in

flask cultures were not attained in the bubble-lift reactor, due to an air filter clogging, which resulted in decreased aeration twice during the experiment.

Table 4.1: Comparison of shake-flask cultures and 50 L bubble reactor removal efficiencies, pH increase, laccase synthesis and biomass synthesis

	Initial shake-flask experiment in Chapter 3	shake-flask experiment in Chapter 4	50 L bubble-lift reactor
COD removal efficiency	60 %	79 ± 1.1 %	62 %
Total phenol removal	83 ± 3 %	80 ± 4.6 %	67 %
Colour removal efficiency	84	71 ± 1.6 %	61 %
Final pH	Not measured	6.1	7.3
Laccase synthesis	2948 ± 90	4550 ± 350	12300 U/l
Greatest biomass	3.2 ± 0.2	7.2 g/l	7.9 g/l

The results for the various removal efficiencies by *T. pubescens* in the brandy distillery wastewater varied with each of the three experiments. COD removal efficiencies were better for the second shake-flask culture, presumably as the stock culture used for inoculation had been pre-acclimatised to a 10 % concentration of the wastewater. It was less efficient in the 50 L reactor due to the aeration problems. Total phenol removal efficiencies were similar in the two shake-flask cultures but lower in the 50 L reactor, again attributable to the aeration problems. Colour removal did not appear as great in the second shake-flask experiment. This could have been possibly been due to a less acidic pH (unfortunately the pH was not measured in the initial experiment) or that slightly less phenols were removed in the second experiment. The removal efficiency in the bubble-lift reactor was again slightly lower. The pH measurements for the second shake-flask experiment and the 50 L reactor were found to be much less acidic after treatment. Unfortunately pH measurements were not taken in the initial shake-flask experiment. Laccase synthesis improved in the second flask culture experiment and improved in the 50 L reactor, even with the aeration problems. The greatest biomass measurements were very similar for the second flask experiment and the 50 L reactor, but nearly half that in the initial shake-flask experiment. This can possibly be attributed to a longer lag phase with the inoculum that had not been exposed to the wastewater and also to the fact that the only measurement taken in the initial experiment was a final reading (i.e. where cell mass would have decreased due to autolysis).

The maximum laccase activity in the shake-flask cultures was just under 5000 units/l, while maximum synthesis in the 50 l reactor was over 12 000 units/l. This was a considerable concentration of a valuable enzyme produced in a simple bubble-lift reactor using a wastewater having had only its pH modified. Although laccase was produced in high concentrations, the wastewater treatment performance was less effective than had been achieved in the flask studies due to a flaw with the air filtration system. Due to moisture collecting in the air filter there were two periods of inefficient oxygen transfer and mixing, which detrimentally affected the aerobic treatment system. The experiment could unfortunately not be repeated because the reactor was on loan for a limited period of time, and the volume of wastewater donated was sufficient for one trial. The scale-up results are very promising but cannot be considered conclusive.

This chapter demonstrated that the biological principles proven in shake-flasks could be scaled up to a 50 l unit on the lab bench. However, further investigation is required before fungal treatment could be considered as a potential treatment method for a range of winery and distillery wastewaters, so following chapters deal with the application of this fungus to a number of different wastewaters. Chapter 5 compares fungal and enzymatic remediation of five wine-related distillery wastewaters and a wine lees, as the addition of a commercially available enzyme represents a simpler treatment method than maintenance of a fungal culture. Chapter 6 assesses the ability of the fungus to remediate a distillery wastewater generated during Amarula production, a situation unique to South Africa, while Chapter 7 investigates fungal treatment as a pre-treatment step for winery wastewaters.

Chapter 5

Fungal and enzymatic treatment of five distillery wastewaters and a wine lees

5.1 Introduction

The alcohol fermentation industry is divided into three main categories: brewing, distilling and wine manufacture. Major difficulties in treating these wastewaters biologically are their variations in the concentration and type of organic compounds, flow volumes and inorganic constituents. Each of these categories produces wastewaters with common characteristics, such as acidic pH values and high BOD and COD (Thassitou and Arvanitoyannis, 2001). A variety of wastewaters may be produced from distilleries using wine-related feedstocks. Various low-grade wines may be distilled to remove the ethanol fraction. Red wine may be distilled to increase the concentration of ethanol and volatile organic compounds to produce brandy, resulting in a wastewater commonly known as rebate. The name is derived from the base wine used in the production of brandy that is known as rebate wine. Wine lees is the sediment or deposit that forms in the bottom of wine casks during the fermentation process. It is rich in tartaric acid and tartrates, which may be extracted, after which the remaining ethanol may be removed by distillation. The colour of wine distillery wastewater is generally attributed to phenolic compounds, although in some distillery wastewaters, such as those derived from molasses, the brown colour can be attributed to nitrogenous polymers known as melanoidins. These arise from the Maillard reaction where complexes form from condensation reactions between reducing saccharides and amines or amino acids (Cämmerer and Kroh, 1995). Distillery and wine-related wastewaters contain high concentrations of phenolic compounds, with literature values ranging from 29 to 766 mg/l (Bustamante *et al*., 2005). Certain phenolic compounds have been implicated in the inhibition of biological treatment by anaerobic digesters (Borja *et al*., 1993a; 1993b).

A review by Coulibaly *et al*. (2003) summarised a number of wastewater characteristics that could adversely affect a biological treatment process. These include temperature, pH, salts, inhibitory molecules (sulphur compounds, surfactants, heavy metals, bleaching chemicals) and the lack of a sufficient carbon and nutrient supply in raw wastewaters. Pretreatment by aerobic degradation of these wastewaters may aid and even improve a second biological treatment step. Mayer (1991) compared biological treatment of a brewery's wastewaters and found anaerobic treatment to decrease the COD concentrations by 91 % at organic loading rates of up to 20 g COD/(l.day), while aerobic treatment resulted in a 76 % removal at a

loading rate of 69 g COD/(l.day). This indicated that an aerobic system would be best suited as a pretreatment step in a remediation process, as it is capable of high COD removal efficiencies under high loading rates.

The presence of heavy metals can seriously hamper bioremediation efforts. Although white-rot fungi require trace amounts of essential heavy metals such as cadmium, manganese or zinc for growth, these metals are toxic when present in excess (Baldrian, 2003). The effect of heavy metals on different species varies, but higher concentrations can inhibit growth, cause morphological and physiological changes and affect reproduction. Although the toxicity of some heavy metals such as mercury, copper or nickel has been used in the development of antifungal wood preservatives, copper is vital to laccase synthesis. Copper is known to be one of the most potent inducers of laccase and has been shown to regulate laccase synthesis on a transcriptional level (Galhaup *et al*., 2002a; 2002b). This would be expected, as it is an essential component of the metallo-enzyme.

Laccase treatment of the phenolic compounds in the wastewaters must also be considered in terms of both phenolic concentrations and structure. The structure of the compound and placement of various side groups affect the kinetics of electron abstraction by laccase as well as the stability of free radicals formed. The tendency of these compounds to be oxidised can be measured using electrochemical methods such as cyclic voltammetry (CV) and differential pulsed voltammetry (DPV). Cyclic voltammetry is the less sensitive of the two methods, but allows oxidation as well as reduction curves to be ascertained, which helps to determine the reversibility of an oxidation reaction. While DPV is more sensitive, it only provides data concerning the oxidation of the selected compound. Mediators are low molecular weight compounds that can be oxidised by laccase to form stable radicals which in turn can oxidise other compounds that cannot be oxidised by laccase directly (Camerero *et al*., 2005). Without the presence of mediators, laccase can only oxidise certain phenolic compounds with an oxidation potential (E^o) ranging from 500 to 800 mV (Camerero *et al*., 2005).

The remediation of wine lees was studied to allow for comparison of the wastewater treatment prior to and after distillation. Although wine lees is a wine-related wastewater it is seldom a

problem as it is rarely released without further processing. It contains compounds of value, such as tartaric acid and ethanol, which are extracted or removed by distillation. This extraction and distillation produces a relatively concentrated, recalcitrant wastewater that is of concern when it does arise as it must be treated before being discharged into the municipal sewer or environment.

5.2 Objectives

The objectives of this study were to investigate the use of *Trametes pubescens* to lower the COD and total phenol concentrations, to increase the pH and to decrease the colour of a variety of wine-related wastewaters. Laccase synthesis was monitored concurrent to bioremediation. The effects of crudely purified laccase were assessed to determine whether it alone would be effective to treat wine-related wastewaters, independently of the fungus.

5.3 Materials and methods

5.3.1 Wastewater samples

Five distillery wastewaters were collected near to Worcester in South Africa in March and May 2006. Two of the distillery wastewaters were obtained from brandy distillation (B1 and B2) and two from spirits distillation (removal of the ethanol fraction) using low-grade wine (S1 and S2). The fifth distillery wastewater was generated from wine lees that had had its tartaric acid extracted and its ethanol removed by distillation (DL). The sixth wastewater tested in this chapter was a wine lees (L) that was obtained from a winery near to Stellenbosch in March 2006. All five wastewater samples were stored at 4 °C. Their colour, pH, electro-activity, COD, phenolic compounds, iron, copper and lead concentrations were measured as described in section 5.3.4.

5.3.2 Flask cultures

The pH of the wastewaters was adjusted to 4.5 using Na_2CO_3 (Saarchem, Merck). Aliquots of 65 ml were placed in 250 ml Erlenmeyer flasks, covered with aluminium foil (to prevent contamination) and autoclaved . The wine lees samples were autoclaved individually in tightly sealed Schott bottles to prevent loss of volatile compounds, and then aseptically transferred to sterilised flasks. Distilled water containing 5 mM succinic acid (Saarchem,

Merck) / lactic acid (Saarchem, Merck) at pH 4.5 served as the control flasks. Flasks were inoculated with *T. pubescens* MB 89 (0.082 ± 0.01 g dry mass) from stock cultures that had been exposed to a 10 % dilution of a distillery wastewater (sample S1) containing 2 % malt extract, 1 % glucose and 0.2 % yeast extract (all Merck, as in section 3.3.1). The samples were placed in a shaking incubator (Labcon) at 150 rpm at 28 °C for twelve days. Wastewater sample flasks were conducted in triplicate. Subsamples were taken daily from each flask and centrifuged in 1.5 ml Eppendorf containers at 9660 *g* for two minutes (Heraeus Biofuge, Germany). The supernatant was aspirated, diluted appropriately and tested for laccase activity, total phenolic compounds concentration, COD, pH, and colour as indicated by a change in absorbance at 525 nm using methods described in section 5.3.4. Fungal dry mass and suspended solids masses were determined using Standard Method 2540D (APHA *et al.*, 1998).

5.3.3 Laccase purification and testing

Laccase was produced in a shake-flask culture in brandy distillery wastewater (sample B1) at pH 5.0 at 28 °C shaken at 150 rpm. After ten days the biomass was removed by centrifugation and filtration. Laccase was precipitated at 4 °C by saturation with ammonium sulphate (Saarchem, Merck). The precipitate was dialysed in 1 mM succinic acid / lactic acid at pH 4.5 over a three day period, with 12-hourly changing of dialysis water. Dialysed materials were combined, centrifuged and filtered through Whatman no. 1 filter paper. The light yellow filtrate obtained had a laccase activity of 4200 units/l. Duplicate Erlenmeyer flasks (250 ml) containing 65 ml of wastewaters at various concentrations at pH 4.5 were spiked such that the final laccase concentration was 25 units/l. These flasks were incubated at 28 °C on a shaker (Labcon) at 150 rpm for 48 hours. Subsamples were taken after 3, 6, 24 and 48 hours and tested for total phenolic compounds, colour and pH.

5.3.4 Analytical methods

The methods for COD concentration, laccase activity and total phenolic compounds concentration determination have been detailed in section 3.3.4. The colour was measured by mixing a 150 µl sample with 150 µl phosphate buffered saline (pH 7) such that the final concentration was 20 mM and measured at an absorbance at 525 nm (PowerWave$_x$, Bio-Tek

Instruments Inc, Winooska, VT, USA). Values were converted to a percentage of the raw wastewater at pH 4.5.

Dissolved iron, copper, lead, cadmium and tin concentrations were measured using atomic absorption spectrophotometry (AAS) (GBC 909 AA, GBC, Australia). Full strength wastewaters were acidified with hydrochloric acid (Merck), filtered (0.22 µm nylon filters Micron Separations Inc), using apparatus that had been acid washed and thoroughly rinsed in deionised water. Standard curves were obtained using appropriate dilutions of 1000 ppm AAS standard solutions of the metals in 1 N nitric acid (EC Lab Services, Port Elizabeth).

The CV and DPV results were obtained using an Autolab PGSTAT 30. A 3 mm glassy carbon electrode (GCE) was used as the working electrode, while an Ag/AgCl electrode (3 M KCl) served as a reference electrode. Platinum wire was used as the auxiliary electrode. All CVs were performed at a scan rate of 50 mV/s. The GCE was cleaned thoroughly before use and between scans by polishing on a Buehler pad with alumina, rinsing with deionised water, rinsing with dilute HNO_3 and finally rinsing with deionised water again. The electrochemistry was performed in freshly prepared Britton-Robinson buffer at pH 4 (adjusted using 2 M NaOH). Britton-Robinson buffer (5 ml) was pipetted into the electrochemical cell and a scan was performed to obtain the base-line. The GCE was removed and cleaned, 20 µl of sample was added and another scan was performed in the stirred solution. A peak was observed if any electro-active compounds were present.

5.4 Results and Discussion

5.4.1 Wastewater characterisation

The results for the characterisation tests are shown in Table 5.1. The distillery wastewaters varied in COD concentrations from 10.5 to 45.5 g/l, while total phenolic compounds concentrations ranged from 35 to 540 mg/l. All but one of the pH values were below 4. No cadmium or tin were found in any of the samples. Copper was found in relatively high concentrations in the two wastewaters from the brandy distillery (22 mg/l in B1 and 15 mg/l in B2). Iron was found in brandy distillery wastewater B2 at a concentration of 14.5 mg/l and in the distilled wine lees that had had its tartaric acid extracted (DL) at 6.7 mg/l. When the

distillery wastewaters tested here are compared to a wastewater derived from molasses spent wash (MSW) they have similar pH values (between 4.0 and 4.3), but the MSW had a much higher suspended solids (2.0 to 2.5 g/l) and COD (92 to 100 g/l) and a BOD of only 52 to 58 g/l (Nandy *et al*., 2002). A much higher proportion of the COD of the wastewaters treated in this study was digestible biologically (shown later). The wine lees had an extremely high total phenolic compounds concentration (1720 mg/l) and COD (212 g/l). A large portion of the COD was attributable to ethanol. Wine lees had the lowest concentrations of all metals. This could be ascribed to the fact that it was the only non-distillery wastewater, and was not heated to boiling point in metal stills or pumped at high temperatures through metal piping.

Table 5.1: Results for wastewater characterisation tests.

	Fe (mg/l)	Cu (mg/l)	Pb (mg/l)	pH	COD (g/l)	Colour (visual)	Total phenols (mg/l)
B1	1.14	21.86	0.15	3.75	29.5	Dark yellow	280
B2	14.47	15.54	0.07	3.90	10.5	Yellow	35
S1	2.75	5.63	0.18	3.67	19.9	Red	320
S2	3.03	0.17	0.11	3.58	34.8	Red	290
DL	6.70	1.69	0.24	5.09	45.5	Brown	540
L	1.02	0.12	0.00	3.72	211.8	Purple	1720

5.4.2 pH

One of the fundamental attributes of a wastewater that affects its treatment is the pH. In biological systems an extreme pH value will prevent treatment with both aerobic and anaerobic processes. Even physicochemical processes are highly reliant on a particular pH for greatest effectiveness. White-rot fungi are more adept at treating wastewaters under slightly acidic conditions than their bacterial counterparts, such as the methanogenic bacteria, which have an optimal pH range between 7.0 and 7.5. Systems incorporating other treatment processes are also pH dependent. Enzymes are known to have optimal pH values and often display a bell-shaped curve when enzyme activity is plotted against pH for a particular substrate. Some enzymes may even display more than one peak when plotted against pH. Optimal activity for laccase from *T. pubescens* MB 89 has been shown to vary from 3.0 to 4.5, depending on the substrates oxidised (Galhaup *et al*., 2002a) and generally has bell-shaped curves when relating activity to pH. The pH values of all the wastewaters tested were adjusted to 4.5. This was to enable a comparison between the enzyme and the fungal culture by using a pH at which the crudely purified enzyme would still have high catalytic activity and the growth of the fungal culture would not be too inhibited.

5.4.3 Chemical oxygen demand removal

Initial experiments were conducted to determine the wastewater concentrations that did not prove toxic to the white-rot fungus by testing shake-flask cultures in 100, 75, 50 and 25 % concentrations of all wastewaters. Once the inhibitory concentrations were established more appropriate concentrations were focused upon. With wine-related distillery wastewaters the COD and colour removal are crucial to bioremediation. The high proportion of easily degradable components in the wastewater can lead to eutrophication of receiving waters if released untreated. High salt concentrations can decrease the availability of oxygen to aerobic organisms as well as affect the osmocity. The COD results for fungal treatment after twelve days are shown in Figure 5.1.

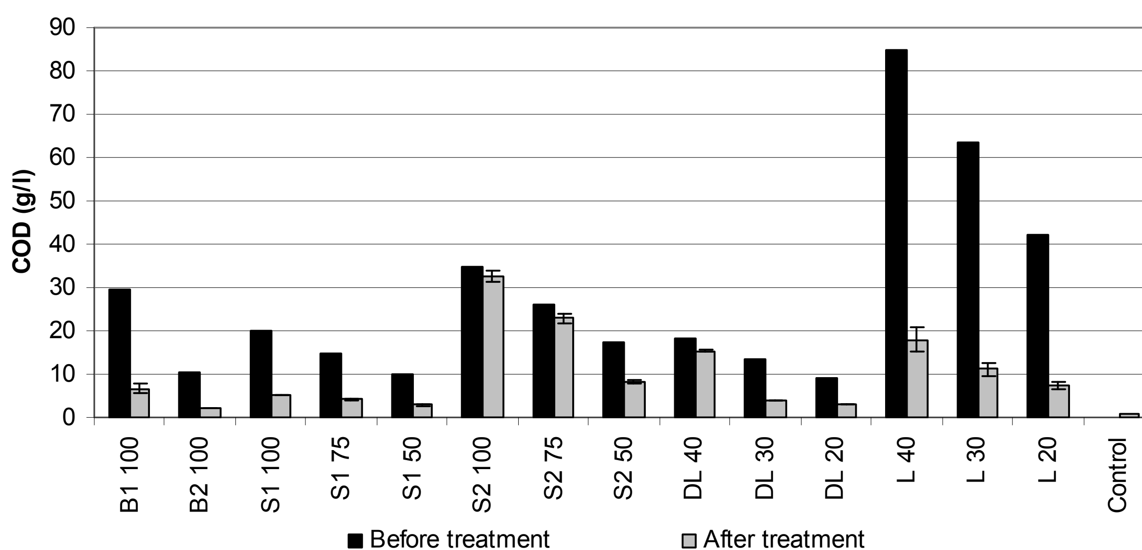


Figure 5.1: Wastewater COD values before and after fungal treatment for wastewaters at percentage concentration. Error bars represent standard deviation and in some cases are too small to be visible (*n*=2).

The two brandy distillery wastewaters (samples B1 and B2) and one of the spirits distillation wastewaters (S1) were easily treated at full strength and had COD removal efficiencies in excess of 70 %. This is comparable to results obtained by González Benito *et al.* (1997), who conducted laboratory batch tests to determine the ability of *Trametes versicolor* to treat molasses-based distillery wastewater. Conditions such as pH, nutrients and carbon source concentrations were tested in order to establish their relation to COD, colour and ammonium removal. González Benito *et al.* (1997) managed to obtain a 77 % COD removal with additional sucrose and KH_2PO_4. One wastewater sample from spirits distillation (S1) was

easily treatable at 100 % strength, while the other proved inhibitory at 75 and 100 % strength (S2). A component of the COD may have inhibited the fungal culture, as the raw COD value was 57 % higher for wastewater S2 than S1. The results here compare well to results obtained by Jiménez *et al*. (2003) who studied the effectiveness of three *Penicillium* spp. and *Aspergillus niger* at treating beet molasses alcoholic fermentation wastewater. The four fungi they tested removed a maximum of 52 % of the COD.

Two of the wastewaters (DL and L) had to be diluted to below 50 % to allow for fungal remediation. The wine lees displayed no COD removal at 50 % strength, but was very well degraded at concentrations of 40 % or less. This may have been attributable to high concentrations of electro-active phenolic compounds, which even at a 50 % concentration were considerably higher than in any of the distillery wastewaters (shown in Table 5.2).

Table 5.2: Results from differential pulse voltammetry and cyclic voltammetry in relation to total phenols.

WW (Total phenols)	CV vs. Ag/AgCl.	DPV vs. Ag/AgCl.
B1 (390 mg/l)	A small peak occurs at 0.4 V.	Small peaks at 0.38 V and at 0.8 V indicating low concentrations of electro-active compounds.
B2 (35 mg/l)	A small peak occurs at 0.4 V.	Peaks at 0.38 V and one at 0.68 V, but at very low concentrations.
S1 (320 mg/l)	There are two peaks, indicating two electro-active compound present in this sample.	There is a highly electro-active compound at 0.37 V. Another peak at 0.8 V is only detectable at higher concentrations.
S2 (220 mg/l)	Small peak at 0.395 V.	Small peak at 0.38 V. More than one compound (due to the width and the bump on the right of the peak).
DL (540 mg/l)	The lack of any distinguishable peaks indicates no electro-active compounds were present.	There is an electro-active compound 0.8 V but it is only evident at higher concentrations.
L (1720 mg/l)	Major peak at about 0.4 V and high concentrations of electro-active compounds at higher potentials.	Very broad peak at 0.38 V. This sample contained highly electro-active compounds.

Phenolic acids have been shown to affect fungal growth rates. Fitzgibbon *et al*. (1998) observed vanillic acid to affect the growth of *Trametes versicolor, Phanaerochaete chrysosporium*, *Mycelia sterilia* and *Geotrichum candidum* to varying extents. Gallic acid did not affect *G. candidum* growth rates, but affected the growth rate of *T. versicolor, P. chrysosporium* and *M. sterilia*. In the wine lees growth inhibition may have been the result of ethanol in the wastewater. At higher concentrations ethanol has been shown to inhibit growth

of *Pycnoporus cinnabarinus.* Fungal radial growth was halved when 20 g/l ethanol was added to the agar culture medium. At 30 to 50 g/l fungal growth was inhibited for 2 to 10 days and the subsequent radial growth rate was decreased by 50 to 70 % (Lomascola *et al*., 2003). At lower concentrations ethanol can actually prove advantageous with fungal treatment systems, as it can serve as a carbon source and an enzyme inducer. Ethanol at a concentration of 3.5 g/l enhanced laccase synthesis tremendously with *P. cinnabarinus* in the study by Lomascola *et al*. (2003).

Distilled wine lees (DL) wastewater proved to be the most difficult to treat and an adequate COD decrease was only achieved when the wastewater was diluted to 30 % strength. A variety of potential factors could have affected the fungus' ability to degrade the wastewaters and these have been described in the introduction to this chapter. The most probable reasons for fungal inhibition were related to toxic concentrations of components of the COD or possibly inorganic ion effects. Different fungal genera have differing tolerances to wastewaters. When Fitzgibbon *et al*. (1998) studied the effect of molasses spent wash concentration on fungal growth rates they observed *G. candidum* and *P. chrysosporium* growth rates increased in the presence of increasing concentrations of molasses spent wash up to 50 %, while the growth of *M. sterilia* and *T. versicolor* was inhibited at concentrations above 5 %. It is not uncommon for industrial wastewaters to inhibit biological treatment. Passarinho *et al*. (1998) observed a yeast (DER 101) able to grow in and completely degrade relatively high concentrations of caffeic acid, vanillic acid, *p*-coumaric acid, gallic acid and catechol (3, 12, 8 and 1.5 g/l respectively) but when it was inoculated into several different olive mill wastewaters (OMW), the maximum degradation of the total phenolic compounds was only 34 %; this was attributed to the presence of other inhibitory compounds.

It should be noted that the pH of the wastewater also makes a large difference to the culture's ability to remain metabolically active. Prior work observed no fungal remediation of Amarula wastewater to occur at the wastewater's original value pH value of 3.9, while it had occurred when the pH was raised to 4.5. A small increase, from pH 4.5 to 5.0, or a larger starter inoculum may have considerably increased the concentrations at which the fungal culture would have achieved reasonable bioremediation efficiencies.

5.4.4 Cyclic voltammetry and differential pulse voltammetry

The results for the CV and DPV are briefly summarised in Table 5.2 and the voltammagrams can be seen in Appendix B. Cyclic voltammetry and DPV data for phenolic compounds were very helpful in that they gave a good indication as to the probability that the compounds would be susceptible to oxidation by laccase. The wine lees had a total phenolic compounds concentration that was three times greater than the highest distillery wastewater, and had a far greater concentration of electro-active compounds.

Of the distillery wastewaters, S1 had the greatest amount of electro-active compounds, followed by S2 and some electro-active compound was evident in brandy distillery sample B1. The second brandy distillation wastewater (B2) contained electro-active species, but they were present at very low concentrations. Wastewater DL displayed no distinct peaks in the cyclic voltammogram other than a small, concentration dependent peak at 0.8 mV vs. Ag/AgCl. This indicated that even though DL contained the greatest concentration of total phenolic compounds of the distillery wastewater samples, it had no electro-active compounds and the phenolic compounds present would probably have the lowest tendency to be substrates for laccase oxidation.

5.4.5 Total phenolic compounds

As can be seen in Table 5.3 the concentrations of phenolic compounds in the samples ranged from as low as 31 mg/l to as high as 566 mg/l. The colours of the wastewaters ranged from light yellow (B2), dark yellow (B1), brown (DL), red (S1 and S2) to dark purple (L). The varying colour and concentrations of phenolic compounds indicated that the phenolic compounds' concentrations and composition varied widely; this was in agreement with results obtained using CV and DPV, which also indicated substantial variations in phenolic compounds concentration and electro-activities.

The results from the enzymatic treatment showed that there was rapid degradation of phenolics (28 to 52 % removal) within three hours for the samples that were red to purple. The degradation of phenolic compounds in the yellow to brown samples only ranged from 14 to 26 %, indicating that the phenolic compounds were not as susceptible to non-mediator

assisted enzymatic degradation. After 48 hours there was appreciably greater relative phenolic degradation in the yellow to brown samples (26 to 45 %), indicating that slower degradation kinetics were involved. Increased degradation was evident in the red to purple samples over the first three hours (ranging from 40 to 61 %) and the majority of the degradation had occurred relatively quickly. This was attributable to the compounds in the red to purple samples containing more electro-active compounds (i.e. with lower oxidation potentials). The wine lees wastewater (L) had an extremely electro-active component while both of the brandy distillation samples and the distilled lees had very low electro-active compounds concentrations. Although the distilled lees sample (DL) had a relatively high concentration of total phenolic compounds, they were shown to have a much higher E^o value than those in the other samples (i.e. less likely to be oxidised by laccase).

Table 5.3: Original total phenolic compounds concentrations and percentage enzymatic and fungal removal.

Sample and concentration	Original [TP] ± SD^3	Laccase treatment		Fungal treatment	
		3 hours ± SD^2	48 hours ± SD^2	48 hours ± SD^3	12 days ± SD^3
B1 100	280 ± 1.8	26 ± 0.5	33 ± 0.3	46 ± 3.4	79 ± 2.2
B2 100	31 ± 4.2	14 ± 0.8	45 ± 4.9	33 ± 5.4	48 ± 7.8
S1 100	315 ± 0.8	46 ± 0.8	52 ± 0.5	82 ± 4.1	83 ± 0.3
S1 75	249 ± 0.3	42 ± 3.3	52 ± 0.6	80 ± 3.5	81 ± 4.4
S1 50	168 ± 0.7	44 ± 1.6	49 ± 1.6	86 ± 1.5	81 ± 1.5
S2 100	301 ± 2.9	33 ± 1.1	41 ± 0.3	47 ± 2.4	61 ± 1.3
S2 75	228 ± 0.9	33 ± 0.6	41 ± 0.4	54 ± 2.7	72 ± 4.2
S2 50	149 ± 0.9	28 ± 1.5	40 ± 2.4	67 ± 5.2	76 ± 1.5
DL 40	223 ± 1.9	16 ± 1.4	26 ± 1.8	30 ± 2.4	60 ± 2.5
DL 30	152 ± 1.5	16 ± 0.8	28 ± 1.6	48 ± 2.6	71 ± 1.7
DL 20	112 ± 6.6	20 ± 0.4	27 ± 2.4	77 ± 4.3	71 ± 1.1
L 40	566 ± 1.7	51 ± 0.6	61 ± 0.5	68 ± 3.1	87 ± 1.6
L 30	436 ± 2.2	52 ± 0.8	60 ± 0.3	70 ± 1.1	78 ± 1.0
L 20	292 ± 4.4	47 ± 0.7	54 ± 0.5	71 ± 2.1	81 ± 1.0

SD^x: Standard deviation$^{\text{number of replicates}}$

[TP]: total phenolic compounds concentration in mg/l

Gökmen *et al*. (1998) also investigated the effects of laccase treatment on removal of phenolic compounds and colour from apple juice and found laccase treatment to increase the polyphenol removal efficiency. Tsioulpas *et al*. (2002) later tested 100 to 1000 units of commercial laccase for phenolic compounds removal from 10 ml samples of diluted OMW and reported that after 24 hours the maximum removal of phenolic compounds was 41 to 44 %. The results obtained in this study made use of laccase at a concentration of 25 units/l and still managed to obtain phenolics removal between 40 to 61 %. Similar enzymatic removal of phenolic compounds from must and wine using immobilised laccase from a

mutant strain of *T. versicolor* has been reported at laboratory scale (Brenna and Bianchi, 1994). Various studies have been carried out in the beverage industry based on the premise that less stable polyphenols are responsible for haze formation in final products. Pretreatment steps using laccase have shown promise with white wines (Minussi *et al*., 2007), while with apple juice laccase contributed to haze formation (Gökmen *et al*., 1998).

The fungal treatment displayed much greater removal of phenolic compounds than the enzymatic treatment alone. From Table 5.3 it was evident that the fungal treatment achieved greater phenolics removal after 48 hours for every sample and dilution than the enzymatic treatment. The phenolics removal efficiencies ranged from 33 to 77 % for the yellow to brown samples and 47 to 86 % for the red to purple samples. After a twelve day digestion the degradation efficiencies had increased to between 48 and 79 % for the yellow to brown samples and between 61 and 87 % for the red to purple samples.

Two of the samples (L and DL) were inhibitory to fungal growth at full strength and had to be substantially diluted to enable treatment. The wine lees (L) had an extremely high total phenolic compounds concentration and was also known to contain ethanol, both of which can inhibit microbial metabolism. Sample DL had the highest total phenolics and COD concentrations of the distillery wastewaters and may have contained a number of inhibitory compounds. The poorest results for removal of phenolic compounds were obtained with the distilled lees (DL) wastewater. Although the wastewater contained a high concentration of phenolic compounds, these compounds were found to be very inactive electrochemically. Significantly greater degradation was obtained by the fungal culture compared to the enzyme with DL wastewater. Only 26 to 28 % of the phenolic compounds were removed by the enzyme, whereas 60 to 71 % were removed by the complete fungal system.

The concentration of phenolic compounds in the control flasks in the complete cultures increased from zero to 16 mg/l during the fungal treatment, showing that the fungus had synthesised or released phenolic compounds. These phenolic compounds may have acted as mediators and allowed for much greater removal of the original phenolic compounds present in the wastewater than the treatment incorporating only the enzyme. Alternatively mediators

may be synthesised from the breakdown of more complex phenolic compounds. Mediator synthesis by *P. cinnabarinus* has been demonstrated with lignin degradation (Eggert *et al*., 1996a). Often compounds that are found to be good mediators are also recalcitrant and as, or even more, toxic than the compounds they are intended to remediate. Camerero *et al*. (2005) evaluated a number of compounds and found phenolic aldehydes, ketones, acids and esters related to the three lignin units to be among the best mediators with dye degradation. These included *p*-coumaric acid, vanillin, acetovanillone and methyl vanillate, as well as syringaldehyde and acetosyringone. Syringaldehyde and acetosyringone were particularly promising due to high dye degradation efficiencies and lower cost and toxicity than other mediators tested.

Degradation of phenolic compounds may not necessarily always improve the characteristics of the wastewaters and in some instances may even result in a more toxic waste. Tsioulpas *et al*. (2002) reported that the decrease in phytotoxicity was not proportional to the phenolic removal in OMW treated with some *Pleurotus* spp. when assayed according to the germination index. Accordingly they proposed a new parameter, namely phenol-toxicity index, as they found the remaining phenolics and to or some of the oxidation products of the laccase reaction in the treated OMW to be more toxic than the parent compounds. Additionally, Coulibaly *et al*. (2003) have reported in a review that during anaerobic digestion of dye wastewaters nitrogen-containing dyes are transformed into aromatic amines that are more toxic and mutagenic than the parent molecules.

5.4.6 Colour

The colour of distillery wastewater is another reason why the wastewater can not be released untreated into the environment. Besides the toxicity of some of the phenolic compounds to aquatic organisms, the darkening of the receiving waters is detrimental to photosynthetic microorganisms. The problem is aggravated by the increased darkening caused by the phenolic compounds as the pH is raised. Yellow solutions at pH 4.0 were observed to be black when the pH was adjusted to neutral using sodium carbonate. The best result obtained by the laccase treatment was after 48 hours in a shake-flask culture at pH 4.5. It was a 12 % decrease in colour in the S1 wastewater (Table 5.4) at 100 % strength (third most colour rich

wastewater). Although this laccase is known to display optimal kinetic activities from pH 3.0 to 4.5 (Galhaup *et al*., 2002a), which allowed for significant degradation of phenolic compounds, there was no significant decrease in colour in the majority of the samples during laccase treatment. The colour of wine lees wastewater increased significantly, even though laccase phenolic degradation efficiencies were the highest in these samples (54 to 61 %).

Table 5.4: Colour removal efficiency (%) of enzymatic and fungal treatment (negative values indicate increases in colour).

Sample and dilution	Laccase treatment		Fungal treatment	
	3 hours ± SD^2	48 hours ± SD^2	48 hours ± SD^3	12 days ± SD^3
B1 100	0.5 ± 0.6	-0.4 ± 0.4	80 ± 4.2	86 ± 2.0
B2 100	-1.1 ± 1.6	3.3 ± 4.7	81 ± 3.1	70 ± 6.5
S1 100	0.2 ± 0.7	12.0 ± 0.7	77 ± 0.5	80 ± 5.5
S1 75	-1.4 ± 1.2	7.9 ± 0.3	81 ± 2.1	79 ± 0.6
S1 50	1.5 ± 1.3	1.6 ± 1.5	87 ± 1.0	83 ± 0.0
S2 100	0.0 ± 0.8	3.6 ± 0.8	79 ± 1.3	76 ± 3.9
S2 75	-0.2 ± 1.5	3.3 ± 0.9	78 ± 2.6	84 ± 2.3
S2 50	0.0 ± 1.4	5.0 ± 1.9	73 ± 3.8	74 ± 2.7
DL 40	0.7 ± 1.4	1.7 ± 0.3	67 ± 1.2	77 ± 1.9
DL 30	1.1 ± 0.9	4.0 ± 1.4	71 ± 1.6	82 ± 2.2
DL 20	-0.8 ± 0.0	5.3 ± 1.9	88 ± 4.7	76 ± 5.6
L 40	-61.1 ± 1.7	-152.4 ± 4.4	54 ± 0.8	82 ± 1.6
L 30	-146.0 ± 1.4	-143.6 ± 1.2	61 ± 5.4	88 ± 1.0
L 20	-159.8 ± 4.7	-143.1 ± 3.3	74 ± 1.1	90 ± 0.9

SD^x: Standard deviation$^{\text{number of replicates}}$

The results for colour removal by laccase treatment were poor compared to the complete fungal system. After 48 hours the colour of all samples treated by the fungal cultures had decreased by 54 to 88 % and after twelve days the colour had been lowered by 74 to 90 %. The wine lees, which was the most colour rich of the wastewaters, had its colour increased by 61 % by the action of laccase, but decreased by up to 90 % by the complete culture. This indicated that even though laccase present in the complete culture would have led to a colour increase, the fungal mycelia managed to remove the resulting intermediate degradation compounds further, such that the colour was vastly improved from an initial deep purple to a final bright yellow. This corroborated results obtained by Tsioulpas *et al*. (2002), who observed that several *Pleurotus* spp. strains were able to grow in OMW without any addition of nutrients and any pretreatment other than media sterilisation. The black colour of OMW became yellow/brown and brighter as the strains grew and 69 to 76 % of the phenolic compounds were removed, with lowest phenolic compound concentrations reached after twelve to fifteen days.

Trametes versicolor has been shown to obtain 82 % colour removal efficiency in molasses-based distillery wastewater (González Benito *et al*., 1997), while *Trametes hirsutus* was observed to display strong decolouration of heat treatment liquor (Miyata *et al*., 2000). González *et al*. (2000) tested the ability of a *Trametes* sp. for colour and COD removal in a culture medium supplemented with 20 % (v/v) vinasse. The presence of vinasse in the culture medium stimulated laccase synthesis 35-fold; the increase in laccase activity corresponded to better colour removal, demonstrating the importance of this enzyme with regards to colour removal. Maximum colour removal was 73 % and maximum COD removal was 62 %; these values were obtained after seven days of fungal treatment. Laccase was not tested in isolation, however. These results using the complete fungal system compare well to those obtained by Raghukumar *et al*. (2004). They found *Flavodon flavus* decolourised diluted molasses spent wash by up to 73 % and lowered the polyaromatic hydrocarbon concentration by 68 %, but this was only obtained in a 10 % concentration of the wastewater. The advantage of using a laccase-producing fungus used in the present study is evident when comparing it to fungi that do not produce the enzyme. Three *Penicillium* spp. and *Aspergillus niger* were studied for their effectiveness at treating beet molasses alcoholic fermentation wastewater and only removed 40 % of the colour (Jiménez *et al*., 2003).

In the flask cultures containing only laccase there was formation of dark insoluble phenolic aggregates due to the polymerisation of some of the phenolic compounds. These were clearly visible as suspended solids causing a dark haze and were pelleted by centrifugation. In the fungal cultures the dark haze was removed after two to three days by the fungal mycelial, resulting in a clear light yellow wastewater with only mycelial pellets evident as suspended solids.

5.4.7 Laccase synthesis

With all wastewater samples the only adjustments made were to the pH or to the wastewater concentration. The highest laccase synthesis obtained with these wastewaters under these conditions was with brandy distillery wastewater B1 (see Table 5.5). At 8997 units/l, laccase activity was nearly three times higher than the second highest activity (obtained in full strength S1). The combination of availability of a carbon source in the COD with the potential

inducers (the presence of copper and phenolic compounds) led to an extremely high synthesis of laccase. Galhaup *et al*. (2002b) found glucose as a carbon source at a concentration of 40 g/l to be optimal for laccase synthesis using *T. pubescens* MB 89 in a synthetic media. The other sample from brandy distillation (B2) also had a relatively high copper concentration, but its potentially poor carbon source (due to the low COD) and very low total phenolics concentration may have hampered the synthesis of laccase. The production of laccase for all wastewaters and concentrations in which an activity of greater than 1800 units/l was observed is shown in Figure 5.2. Wastewater S1 had a moderate copper concentration (5.63 mg/l), a fairly high total phenolic compounds concentration (320 mg/l) and an intermediate COD (19.9 g/l). This was enough to allow for the second highest laccase synthesis when tested at full strength. Interestingly, wastewater S1 was the only sample that produced an appreciable concentration of laccase constitutively (associated with growth and primary metabolism). This is evident from the high laccase activity displayed on day two in Figure 5.2.

Table 5.5: Maximum laccase activity, days on which the maximum activities occurred and the relative fold increase compared to the control.

Sample	HLA* (units/l)	Day of HLA	Fold increase
Control	260	12	1
B1 100	8997	9	34.6
B2 100	2847	12	11.0
S1 100	3354	10	12.9
S1 75	2266	7	8.7
S1 50	1491	7	5.7
S2 100	180	3	0.7
S2 75	171	3	0.7
S2 50	1833	8	6.2
DL 40	125	2	0.5
DL 30	2043	7	7.9
DL 20	1650	7	6.3
L 40	2929	10	11.3
L 30	1535	3	5.9
L 20	2133	3	8.2

*Highest laccase activity

Wine lees had an extremely high total phenolics concentration (even at a concentration of 40 %) and contained ethanol, both of which are known to increase laccase synthesis. Unfortunately, the lack of a sufficient concentration of an easily utilisable carbon source coupled to the lowest copper concentration (aggravated further by dilution) may have hampered very high synthesis, but even so an activity just below 3000 units/l was observed in the 40 % concentration. Very low activities were obtained with wastewater DL at or above

40 % and for wastewater S2 at or above 75 %. Wastewater DL had a very high COD concentration for a wine-related distillery waste, even after the tartaric acid had been extracted and the ethanol removed by distillation. The low laccase activities obtained were attributable to fungal growth inhibition, as shown by the poor COD removal efficiencies over the twelve days of digestion, which negated laccase synthesis. Laccase synthesis could not be correlated to electroactivities of the phenolic compounds present in the wastewaters. The weaker brandy distillery wastewater (B2) displayed high laccase synthesis even though it had an extremely low concentration of phenolic compounds. This indicated the importance of copper, even if only at low concentrations, with respect to allowing for greater laccase synthesis. The wine lees had the greatest concentration of electro-active compounds and it was possible that the early stimulation of laccase synthesis displayed in the 20 and 30 % concentrations of the wastewater (highest laccase activities recorded on day 3, see Table 5.5) were induced by these electro-active compounds.

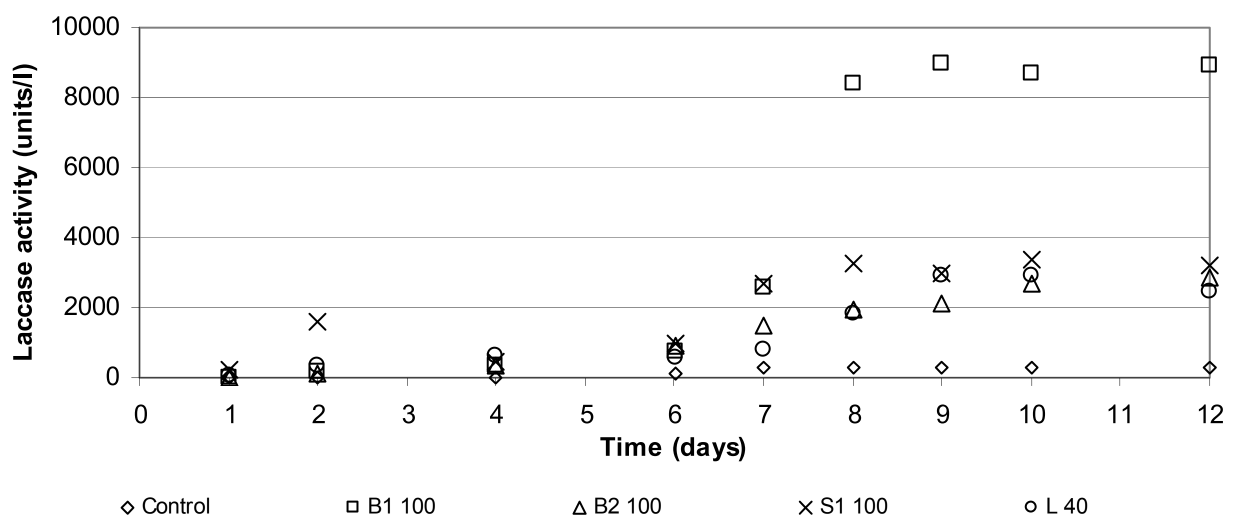


Figure 5.2: Laccase synthesis for wastewaters and concentrations in which *T. pubescens* synthesised laccase at concentrations greater than 2500 units/l and the control.

As mentioned earlier, Tsioulpas *et al*. (2002) obtained a laccase activity of 301.7 ± 8.5 units/l using a *Pleurotus* species in OMW. This was a good result for that fungal genus, but was very low when compared to the results obtained with *T. pubescens* MB 89. These results compare favourably to other agricultural waste residues used to produce laccase, as one distillery wastewater produced 8997 units/l with only its pH modified. Osma *et al*. (2007b) used banana skins as a support substrate for the same strain of fungus and managed to obtain laccase

synthesis of 1570 units/l. Recent work on *Trametes pubescens* only obtained laccase activities of 400 units/l when it was grown on stainless steel sponges in static flask cultures. Mandarin peels served as a carbon source and this was optimised with the addition of soy oil (Osma *et al*., 2007a).

5.5 Interim summary and conclusions

Considerable differences were observed between the various wastewaters with all characteristics that were tested (Table 5.1), yet *Trametes pubescens* MB 89 greatly improved the quality of all six wastewaters. Two wastewaters (wine less and the distilled wine lees) had to be diluted to below 50 % to allow for bioremediation by the submerged fungal culture. The fungal culture displayed much better properties than the enzyme alone in removing both the total phenolic compounds and colour. Fungal treatment resulted in a decrease in the COD of up to 83 ± 2.1 %. The greatest degradation of phenolic compounds by the fungal culture was 87 ± 1.6 % in contrast to 61 ± 0.5 % by laccase alone. Colour removal of up to 88 ± 4.7 % was attained by the submerged fungal culture, while the highest removal by laccase was only 12 ± 1.6 %.

Enzymatic treatment reduced the total phenolic compounds but did little to improve the colour of the wastewaters, and in the case of wine lees significantly increased the colour. The wine lees dilutions contained the highest levels of phenolic and electro-active compounds and displayed the most dramatic results with enzymatic treatment. Although laccase treatment resulted in total phenolics decreases of up to 61 ± 0.5 %, the colour was increased by up to 160 ± 5 %, indicating that the new compounds formed by laccase were more colour rich than the parent compounds. The complete fungal system was found to be superior to enzymatic treatment alone.

Laccase synthesis greater than 1500 units/l was obtained in all wastewaters. A concentration of 8997 units/l was obtained in the rebate wastewater having an initial COD of 29.5 g/l, total phenolic compounds concentration of 280 mg/l and a copper concentration of 21.86 mg/l. Only its pH was modified (from 3.75 to 4.5).

Chapter 6

Characterisation of Amarula distillery wastewater, aerobic remediation using *Trametes pubescens* MB 89 and the subsequent production of laccase

6.1 Introduction

A variety of raw materials such as grapes, potatoes, sweet potatoes, rye, barley and wheat are fermented in order to produce potable ethanol and then distilled in order to produce more potent alcoholic beverages such as brandy, vodka, schnapps, shochu, bourbon and whiskey. The pulp from the fruit of the marula tree is used in the production of Amarula Cream, a liqueur produced in the Republic of South Africa that has been received well internationally. The marula tree (*Sclerocarya birrea*) is dioecious and indigenous to certain warm, frost-free regions of sub-equatorial Africa. The female tree bears a small oval fruit that grows to approximately 3 to 5 centimetres in diameter. It is composed of a tough outer skin enclosing white fibrous flesh and a large stone which houses two to three kernels. The green fruit abscises before ripening, allowing for the ripened yellow fruit to be harvested by collection from the ground. The pulp is separated from the hard kernels of selected fruit and pumped into stainless steel cooling tanks, kept below 8 °C to prevent fermentation and transported in bulk by tankers to the Distell Group Ltd cellar in Stellenbosch in the Western Cape.

At the cellar the pulp is transferred to fermentation tanks, where a pure yeast culture is inoculated into the pulp to start the fermentation process using conditions similar to those of winemaking. Once fully fermented the clear marula wine is transferred to the distillery. The fruit solids are compressed to extract all the juice and then distilled to release the marula fruit flavours, which are added to the marula wine. During fermentation, which is performed at 18 °C to 20 °C, the natural fruit sugar present in the marula is converted to alcohol, taking seven to ten days. During this stage the solid fruit particles settle at the bottom of the tanks. The marula wine is distilled in column stills and then in copper pot-stills to produce the Amarula spirit, which is matured for two years in small oak barrels. The final step in the creation of Amarula Cream is the blending of the liqueur with fresh cream until a smooth consistency is formed, resulting in a cream product that is rich and soft, with an alcohol content of 17 %. There is currently no scientific literature available as to the characterisation of wastewaters originating from marula distillation.

Very little scientific literature is available regarding the marula plant and products derived from it. Marula bark is widely used for bacteria-related diseases by indigenous cultures in

Africa. Eloff (2001) investigated whether the ethnobotanical use could be validated by laboratory studies. All extracts were active with minimum inhibitory concentration values from 0.15 to 3 mg/ml using *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus faecalis* as test organisms. Commercial enzymes have been compared for their ability to improve yield and clarification of marula fruit juice (Fundira *et al.,* 2002). Juice yield was increased by 12 % using Rapidase Filtration, juice clarity was improved by 15 % and total terpenes were increased with prefermentation enzymes, while post-fermentation treatment with enzymes led to an increase in free monoterpenes of 92 %. The enzymes had different effects on the aroma and the flavour of the juice, wine and distillate. Mduli (2005) partially purified a polyphenol oxidase (PPO) and peroxidase from the fruit by a combination of temperature induced phase separation in Triton X-114, DEAE-ion exchange and Sephadex G100 gel filtration. The PPO was purified 58-fold with a 75 % recovery. The PPO and peroxidase shared the same molecular weight (71 kDa) and pI (5.43). Thermal deactivation curves were bi-phasic for both activities. The pH optimum for PPO was 7.0 with catechol. Marula PPO had K_M values of 1.41, 1.43, 3.73 and 4.99 mM for catechin, 4-methylcatechol, 3, 4-dihydroxyphenylpropanoic acid and catechol, respectively.

6.2 Objectives

The objectives of this work were to characterise a distillery wastewater from Amarula production and ascertain the effectiveness of a biological treatment step using aerobic digestion with a monoculture of *Trametes pubescens* MB 89. Additionally the optimal concentration of wastewater that would elicit the greatest synthesis of laccase during bioremediation was to be determined.

6.3 Materials and methods

6.3.1 Wastewater characterisation

Wastewater from Amarula production was obtained from Distell Group Ltd in Stellenbosch and stored at 4 °C. Dissolved iron, copper, lead, cadmium, tin and magnesium concentrations were measured using flame atomic absorption spectrophotometry (AAS) (GBC 909 AA, GBC, Australia). Full strength wastewater was acidified with hydrochloric acid (Saarchem, uniLAB, Merck), filtered (0.22 µm nylon filters, Micron Separations Inc.) using apparatus

that had been acid washed and thoroughly rinsed in deionised water. Standard curves were obtained using appropriate dilutions of 1000 ppm AAS standard solutions of the metals in 1 N nitric acid (EC Lab Services, Port Elizabeth). The nitrate concentration was measured using Spectroquant kit 1.4543.0001 (Merck) analogous to standard method 4500-NO_3^- B (APHA *et al.*, 1998). Total suspended solids (TSS) concentration was determined using a method developed from standard method 2540 D (APHA *et al.*, 1998). The TSS was measured by centrifuging two samples of 1 l of the Amarula wastewater at 22095 *g*. The pellet was washed, resuspended and centrifuged five times in an equivalent of distilled water. The supernatant was filtered (Whatman no. 1) and the retained solids were rinsed with distilled water. The retained solids were combined with the washed pellets from centrifugation, dried at 105 ºC overnight and weighed (APHA *et al.*, 1998). The COD concentration and total phenolic compounds concentration was measured as described in section 3.3.4. The pH was measured using a Cyberscan 2500 electrode (Eutech Instruments, Singapore).

6.3.2 Bioremediation and laccase synthesis

The pH of 100, 80, 60, 40, 20 and 10 % concentrations of the wastewater was adjusted to 4.5 using sodium carbonate powder (Saarchem, uniLAB, Merck Chemicals Pty. Ltd.) and 80 ml aliquots (in duplicate and diluted with distilled water) were autoclaved in 300 ml Erlenmeyer flasks. Distilled water containing 5 mM succinic acid (Saarchem, Merck) and 5 mM lactic acid (Saarchem, Merck) buffer at pH 4.5 served as the 0 % wastewater control. The wastewater was inoculated with actively growing *Trametes pubescens* MB 89 that had been cultured in liquid media containing 2 % malt extract (Biolab, Merck), 1 % glucose (Saarchem, uniLAB, Merck) and 0.2 % yeast extract (Biolab, Merck) on a bench top shaker (Labcon) at 150 rpm at 28 °C. The biomass was separated by centrifugation in a Beckman J-14 rotor (14300 *g* for 1 minute), rinsed in sterile distilled water and centrifuged under the same conditions. The supernatant was decanted and equal portions of the rinsed *T. pubescens* biomass (760 ± 50 mg dry mass/l) were inoculated into each flask and placed on a bench top shaker at 150 rpm at 28 °C for a period of 14 days. Samples (<1.5 ml) were removed every 48 hours and laccase activities, total phenol concentrations, COD concentrations, pH and colour absorbance at 525 nm were monitored. The absorbance at 525 nm was measured by

mixing a 100 µl sample with 200 µl distilled water in 25 mM phosphate buffered saline at pH 7.0. Laccase activities were measured as described in section 3.3.4.

6.4 Results and Discussion

6.4.1 Wastewater characterisation

The wastewater displayed a number of characteristics that would make it difficult to treat using conventional biological systems (shown in Table 6.1). The values in Table 6.1 have been compared to levels that are permissible for land irrigation (DWAF, 1996a) and discharge into a water body (DWAF, 1996b) according to the South African Department of Water Affairs and Forestry (DWAF) and Australian and New Zealand regulations (ANZECC and ARMCANZ, 2000).

Table 6.1: Characterisation of the wastewater (mg/l unless stated otherwise).

Parameter	Value in wastewater	DWAF (1996a)	DWAF (1996b)	ANZECC and ARMCANZ (2000)
pH	3.8 pH units	6.5 to 8.4	Less than 0.5 pH unit or 5%.	
Total phenols	850 ± 16			
Phenol			<30 µg/l	320 µg/l
4-chlorophenol				220 µg/l
2, 4-dichlorophenol				160 µg/l
2, 4, 6-trichlorophenol				20 µg/l
Pentachlorophenol				10 µg/l
COD	26700 ± 310			<40
Colour at 525 nm at pH 3.8	13.1 m^{-1}			
Total suspended solids	10570 ± 410	50	<10 % of background (<100 mg/l)	<40
Iron	1.51	<5		Insufficient data
Copper	0.11	<0.2	<0.3 to <1.4 µg/l	1.4 µg/l
Lead	0.07	<0.2	<0.2 to <1.2 µg/l	3.4 µg/l
Cadmium	0.00	<0.1	<0.15 to <0.4 µg/l	0.2 µg/l
Tin	0.00			Insufficient data
Magnesium	<LOD**			<15
Nitrate	76 ±6			

*Freshwater trigger values at a 95 % level of species protection.
**LOD: limit of detection for magnesium 20 mg/l.

The pH was very acidic and would require neutralisation using a compound such as agricultural lime (calcium oxide) to at least pH 6.5 to allow for use in irrigation (DWAF, 1996a) or up to pH 7.0 for biological treatment (e.g. by anaerobic digestion). The DWAF recommends a target water quality range of 6.5 to 8.4 for irrigation purposes. Below a pH of 6.5 crop foliar damage occurs and the availability of micro and macro-nutrients increases,

leading to long term toxicity. Crop foliar damage also occurs under very alkaline conditions and micro and macro-nutrients are less available. Excessive acidity also results in metal and concrete corrosion, while higher alkaline conditions result in encrustation of irrigation equipment.

The TSS concentration was not only very high, but also included a component that was very difficult to remove due to the size range of the residual pulp in the wastewater. A fine suspension remained even after centrifugation at 22095 *g*, which was only completely removed with filtration through 0.22 μm pore filter membranes used for the preparation of samples for atomic absorption spectroscopy. The DWAF has proposed a target water quality range of less than 50 mg/l TSS for irrigation use. The concentration of suspended solids in this wastewater would render it unsuitable for use in irrigation, as concentrations of more than 100 mg/l lead to severe clogging of drip irrigation emitters. It would also not be permitted to be discharged into a water body, as the regulations stipulate a concentration of less than 10 % variance relative to the background levels, which may not exceed 100 mg/l. Discharge of a high TSS concentration into an aqueous environment results in a decrease in light penetration and thereby a decrease in photosynthesis and primary production, which reduces food availability up the trophic chain. It may also affect filter feeders, gill functioning, lower foraging efficiency due to decreased visibility, abrasion of benthic organisms and the settling out may result in changes of substratum and community population shifts (DWAF, 1996b). Even though this characteristic falls within the treatable parameters of anaerobic digesters, which can cope with a COD : TSS ratio of 1.8 (Metcalf and Eddy, 1991), it is still unlikely to fall within the required limit for environmental discharge without undergoing some form of screening. The suspended solids are lignocellulosic in nature and would not be broken down substantially by anaerobic bacteria.

The concentration of phenolic compounds of 850 mg/l in this wastewater was very high relative to concentrations previously reported in vinasse from wine distillation. Bustamante *et al*. (2005) characterised a number of distillery wastewaters in Spain and found the total phenolic compounds to range from 65 to 766 mg/l with a mean of 318 mg/l. The high level of total phenolic compounds in this wastewater could negatively impact on a traditional

biological treatment system, as this characteristic alone has been shown to adversely affect anaerobic treatment systems (Jiménez *et al*., 2006; García García *et al*., 1997). Anaerobic digestion has been shown to be 50 % less active when exposed to relatively low concentrations of phenol, catechol and resorcinol at 26, 24 and 29 mM respectively (Parkin and Owen, 1986). Phenol is thought to be a nerve poison and possibly results in irreversible damage to protein structure and reduced rates of photosynthesis in aquatic plants (DWAF, 1996b). Unfortunately, the parameter measured, total phenols, does not allow for determination of individual compounds' concentrations. Considering that phenolic compounds originate from an edible fruit, it is unlikely that they are toxic to humans, but at these concentrations in the wastewater they may still be inhibitory to microorganisms. The concentration of phenols would lead to a great increase in colour if the pH were to be increased from 3.8 to neutral. Even at the original pH of 3.8 the colour value is 13.1 m^{-1}, which would affect light penetration should the wastewater be discharged into a water body.

The COD (26.7 g/l) was high and would possibly require either dilution or long hydraulic retention times to facilitate treatment. If released into a water body the large concentration of organic compounds would serve as a nutrient source and facilitate bacterial blooms.

The DWAF (1996a) guidelines for irrigation recommend a total N concentration of less than 5 mg/l to avoid affecting sensitive crops such as grapes and most fruit trees. The DWAF consider total N as the combination of ammonium, nitrate, nitrite and ammonia. Ideally the N level should be low enough such that under normal irrigation levels most of it would be utilised by the crop and not leach into the groundwater. The value measured for nitrates alone puts this wastewater above the stipulated target for irrigation.

The DWAF (1996b) target water quality range for metals varies, according to the hardness of the receiving water body. For copper, the maximum allowed discharge values into a water body vary from 0.3 mg/l to 1.4 µg/l, depending on the hardness of the water, ranging from below 60 to greater than 180 mg $CaCO_3$/l. All the metals that were measured in this study fell within the permissible values under both South African (DWAF, 1996a; 1996b) and Australian and New Zealand guidelines (ANZECC and ARMCANZ, 2000). The metals found

in the wastewater were not present at concentrations that would negatively affect a biological treatment process. The presence of ferrous ions would actually facilitate the removal of phenolic or organic compounds should a treatment process such as Fenton oxidation (catalytic process based on an electron transfer between hydrogen peroxide and a metal acting as a homogeneous catalyst) be used to treat the wastewater.

6.4.2 Bioremediation

6.4.2.1 Chemical oxygen demand

From the results (Figure 6.1) it was evident that the COD removal efficiency of this fungal species under these culture conditions was approximately 75 %. Between 71 and 77 % of the COD was removed by the end of the experiment from all the wastewater concentrations tested. Removal efficiencies were 71, 72, 75, 77, 74 and 71 % for 10, 20, 40, 60, 80, 100 % wastewater concentrations respectively, after fourteen days of fungal digestion. This is comparable to prior results obtained with *T. pubescens* MB 89 where 79 ± 1.1 % of the COD was removed from brandy distillery wastewater (Section 4.4.1.1).

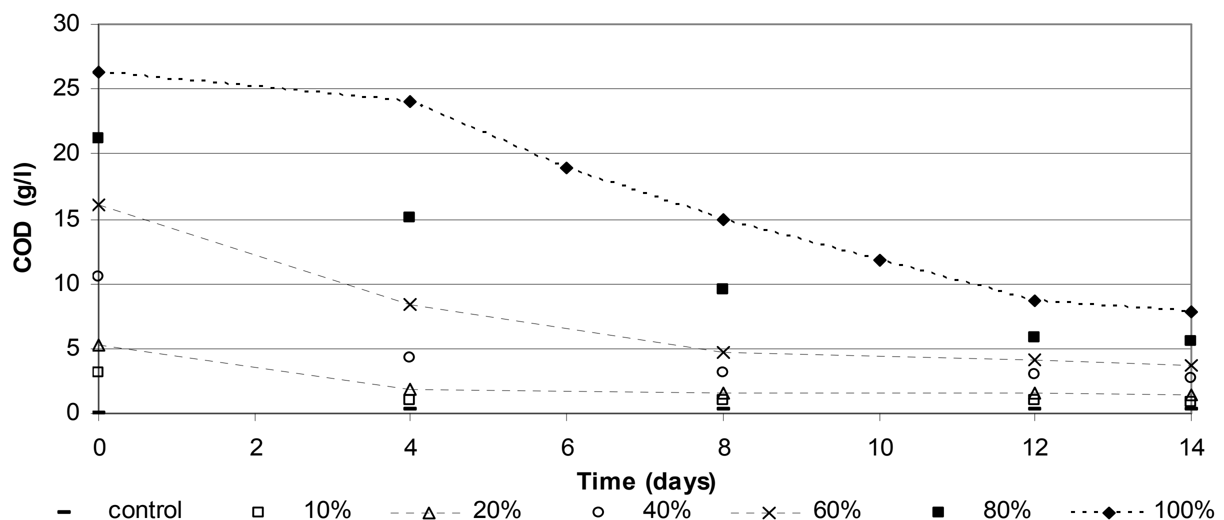


Figure 6.1: Change in COD (g/l) over a 14 day period for various Amarula wastewater concentrations inoculated with *T. pubescens*. Highest standard deviation: 0.86 g/l.

González Benito *et al.* (1997) used *Trametes versicolor* in distilled beet molasses wastewaters that had been subjected to anaerobic-aerobic pre-treatment. They obtained a comparable 77 % COD removal when the wastewater was supplemented with sucrose and KH_2PO_4. This

wastewater was significantly more concentrated and yet *Trametes pubescens* achieved the same COD removal efficiency without requiring any carbon or nitrogen supplementation. The greatest COD removal in 10 and 20 % concentrations occurred within the first four days, after which little further removal occurred. This was not unexpected as the lower biodegradable COD in a lower wastewater concentration would have been consumed more rapidly by the inoculum.

Figure 6.2 illustrates the concentration of COD removed by the biomass by the fourth day after inoculation. The trend that would have been observed if the biomass had not been inhibited would have been either an increasing amount of COD/l removed as the percentage wastewater increased (and thereby potential substrate for removal increased) or a plateau as the maximum removal efficiency for the amount of biomass present was attained. The highest total mg COD removal over the first four days occurred at a 60 % wastewater concentration.

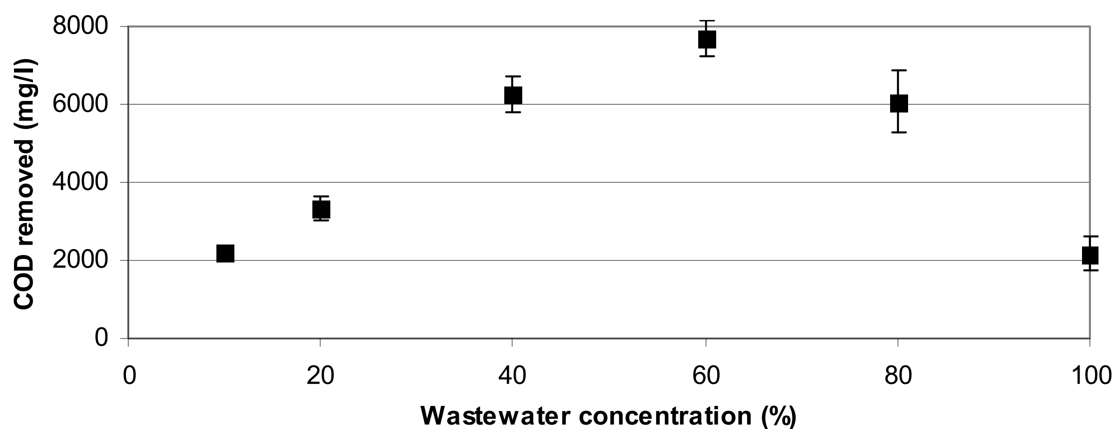


Figure 6.2: Total mass COD removed (mg/l) by day four for various Amarula wastewater concentrations inoculated with *T. pubescens*. Error bars represent standard deviation.

Above 60 % wastewater concentration lower total mg COD removal occurred, indicating that the biomass was initially inhibited by some component in the wastewater, possibly attributable to the greater concentration of phenolic compounds. This was most evident in the 100 % wastewater samples (Figure 6.2). Over the first four days the total mg COD removed from the 100 % wastewater was less than 30 % of the total mg COD removed in the 60 % wastewater concentration over the same time period.

6.4.2.2 Phenolic compounds

The greatest decrease in total phenolic compound concentrations occurred within the first two days after inoculation (Figure 6.3). Further rapid decreases only occurred between days two and four for the two highest wastewater concentrations (80 and 100 %). The data for the 80 and 100 % wastewater on day six (reported later, see Figure 6.6) suggests that more than sufficient laccase was present to oxidise all possible reactant phenolic compounds present. Yet a further 8.4 ± 2.9 % decrease in the concentration of phenolic compounds took place over the next eight days of the experiment, which indicated that a portion of the phenolic compounds was degraded in a manner other than laccase-catalysed oxidation. This trend was also observed in brandy distillery wastewater (section 4.4.1.3).

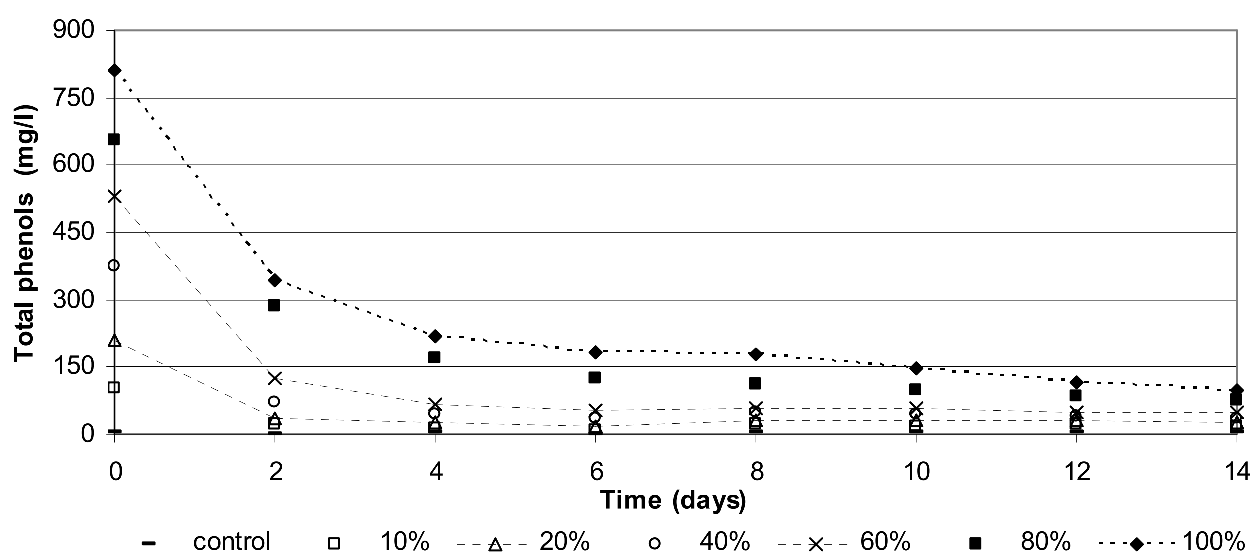


Figure 6.3: Change in total phenolic compounds concentration over a 14 day period for various Amarula wastewater concentrations inoculated with *Trametes pubescens*. Highest standard deviation: 11 mg/l.

The level of total phenol removal efficiency, when considered for the lowest residual phenols value for each of the wastewater concentrations tested, varied only between 87 and 92 %. Removal efficiencies of phenolic compounds after fourteen days of fungal digestion were 83, 88, 90, 91, 88 and 88 % for 10, 20, 40, 60, 80 and 100 % wastewater concentrations respectively. Approximately 10 % of the phenolic compounds were not degradable by the fungal treatment or by laccase. This could be attributed to the residual phenols having an E^o above the oxidation potential of laccase (Camerero *et al*., 2005), the lack of phenolic compounds that were sterically inaccessible to laccase, or the presence of reactive intermediates formed by laccase oxidation. Jiménez *et al*. (2006) also compared anaerobic

digestion of untreated vinasses and pre-treated vinasses (previously fermented with *Penicillium decumbens*) and observed that the combined aerobic/anaerobic process displayed better overall COD removal (96.5 % compared with 90.0 %), a decrease in the hydraulic retention time required and greater colour removal. They attributed the improved process performance, kinetics and stability displayed by the pre-treated vinasses to the lower levels of phenolic compounds.

In this study removal of phenolic compounds from the Amarula wastewater was 10 % greater than the removal from brandy distillery wastewater (80 ± 4.6 % removal) reported in section 4.4.1.3. This was achieved even though the total phenolic compounds concentration was higher in this wastewater (850 mg/l) than it had been in the brandy distillery wastewater (540 mg/l). The results in this chapter also compare well to a study by García García *et al*. (1997), who reported that the fungus *Geotrichum candidum* was able to remove 70 % of the total phenolic compounds from a vinasse from molasses fermentation (raw wastewater value of 469 mg/l). The present study obtained a higher removal efficiency from a wastewater containing a greater total phenolic compounds concentration. Different fungi have different dominant enzymatic pathways and that of *T. pubescens* may be more efficient than that of *G. candidum*. However, different removal efficiencies may be attained with different wastes using the same fungus. Different plant source materials containing different phenolic compounds in differing concentrations and these phenolic compounds will display differing recalcitrance to biological treatment. Amarula, brandy and molasses production utilise very different source materials and consequently have different types of and concentrations of phenolic compounds in their related wastewaters. Phenolic compounds vary in their susceptibility to laccase and to fungal degradation. A higher concentration of more susceptible phenolic compounds would allow for better removal efficiencies.

A variety of laccase-induced reactions occur that result in the degradation of phenolic compounds. The initial free radical formed by laccase oxidation is typically unstable and may undergo a second enzyme-catalysed oxidation such as the conversion of a phenol to a quinone. Phenoxy radicals may also undergo non-enzymatic reactions that may result in polymer degradation (due to the cleavage of covalent bonds linking the monomers), ring

cleavage of aromatic compounds or even covalent crosslinking of monomer radicals to form dimers, oligomers or polymers. The polymerisation reaction can lead to the formation of an insoluble melanin-like product that may precipitate out of solution (Claus, 2004). The formation of a fine, dark, insoluble precipitate was evident in the brandy distillery wastewater shortly after inoculation (section 4.4.1.4). This was an ideal scenario as it indicated the complete removal of the phenolic compounds from the wastewater, in the form of an insoluble, amorphous, polymeric complex. However, even though the concentration of phenolic compounds was reduced by 88 % in both the 80 and 100 % Amarula wastewater concentrations, there was a notable increase in colour, which is discussed in the following section.

6.4.2.3 Colour

The change in colour exhibited a clear trend throughout the concentration range assessed (Figure 6.4). There was an increase in the absorbance reading over the first two days, which coincided with the greatest removal of phenolic compounds, followed by the absorbance gradually decreasing over the remainder of the experiment. The increase was attributable to laccase converting phenolic compounds to more colour-rich compounds, which were probably the quinone version of the original phenolic compounds. The subsequent colour decrease was probably the result of further degradation by the fungal biomass, although it could possibly have been due to natural degradation of unstable compounds resulting from enzyme-catalysed oxidation. Wastewater concentrations below 60 % had a final absorbance 8 to 27 % less than the original absorbance. Conversely, the samples at 80 and 100 % were more colour-rich than the initial untreated wastewater. It should be noted that an increase in pH leads to an increase in the absorption spectrum with certain phenolic compounds. This was visibly evident in all wastewaters that had their pH adjusted, and could also be seen when comparing the colour of winery wastewaters at their initial pH and at the pH for anaerobic digestion in the next chapter. The colour change observed may be attributable to the conversion of the phenol to the quinone version in a hydroxyl-rich solution (by abstraction of the hydrogen cation from the OH group of the phenol).

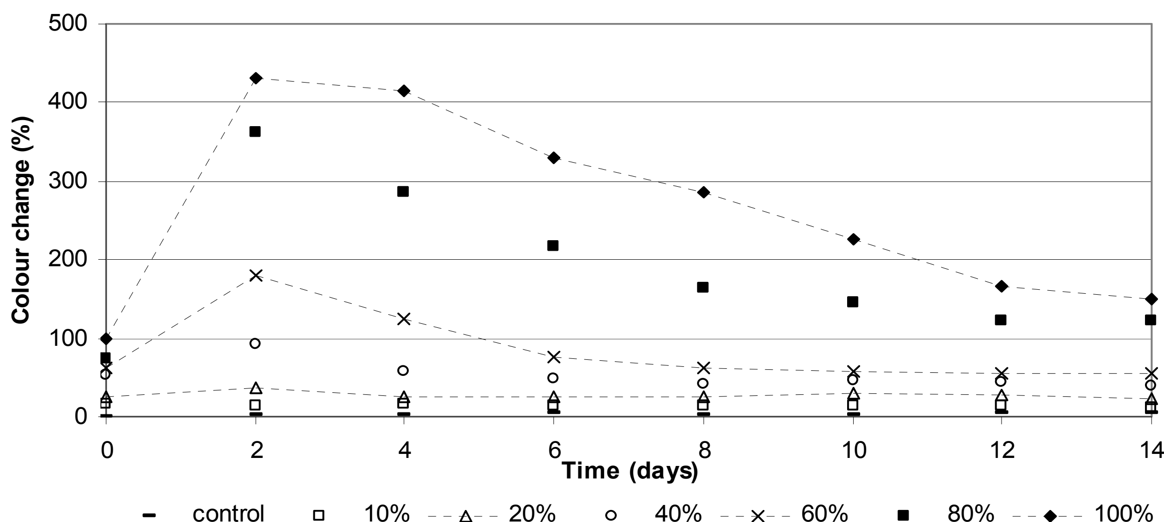


Figure 6.4: Change in colour over a 14 day period for various Amarula wastewater concentrations inoculated with *Trametes pubescens*. Highest relative standard deviation: 14 %.

After fourteen days of fungal digestion the colour removal efficiency was 27 ± 4, 8 ± 5, 26 ± 2 and 10 ± 1 % for the 10, 20, 40, 60 % wastewater concentrations respectively, while the colour increased by 66 ± 17 % and 50 ± 12 % for the 80 and 100 % wastewater concentrations, respectively. This was again attributable to the conversion of the phenolic compounds into compounds that were more colour-rich than the original parent molecules. The addition of mediators could have significantly decreased the colour of the wastewater. Laccase is hampered by its low oxidation potential, but the addition of mediators allows for the oxidation of compounds that have oxidation potentials greater than that of laccase. Mediators work in two manners: mediators can be oxidised by the enzyme and in turn be reduced when they in turn oxidise another compound, or be oxidised and then react with another compound, creating a dimer, oligomer or polymer that in turn can precipitate out of solution with other suchlike reacted compounds. The problem with adding inducers is that they are expensive and can be toxic, which makes their use in pilot scale treatment economically unfeasible.

This chapter demonstrated that *T. pubescens* was capable of treating a high strength distillery wastewater from Amarula production at 100 % concentration. Fitzgibbon *et al.* (1998) compared *Geotrichum candidum, Trametes versicolor, Phanerochaete chrysosporium* and *Mycelia sterilia* for colour removal from molasses vinasse. The greatest removal was obtained with *Trametes versicolor*, which reduced the colour by 53 % after ten days of digestion.

Unfortunately this could only be achieved at a wastewater concentration of 12.5 %, as higher concentrations proved inhibitory to the fungus. Bioremediation in such a case would have proved unfeasible as the considerable dilution required would preclude the use of the treatment system.

6.4.2.4 pH

The lower the wastewater concentration, the greater and more rapid the increase in pH over the fourteen day period with fungal treatment (Figure 6.5). This was as expected, since lower concentrations correspond with lower buffering capacity of the medium. After fourteen days of fungal treatment the pH was 7.76, 6.68, 6.47, 6.03, 5.55, 5.75 and 4.89 for the control, 10, 20, 40, 60, 80 and 100 % wastewater concentrations respectively. The control stabilised at a pH just above 7.5 within six days, while the wastewater samples only stabilised towards the end of the testing period. The control was distilled water with 5 mM succinic / lactic acid buffer and the increase in pH was more than likely attributable to the degradation of the fruit acids. The increase in pH for the various wastewater concentrations was a result of the degradation of the organic acids extracted from the pulp of the marula fruit. A variety of organic acids are present in agro-industrial wastewaters. Tartaric acid, lactic acid and succinic acid have been shown to comprise the majority of the organic acids found in a wine distillery wastewater, while malic acid and acetic acid are also present (Wolmarans and de Villiers, 2002). The marula fruit is known to be a rich source of ascorbic acid (TIFP, 2007).

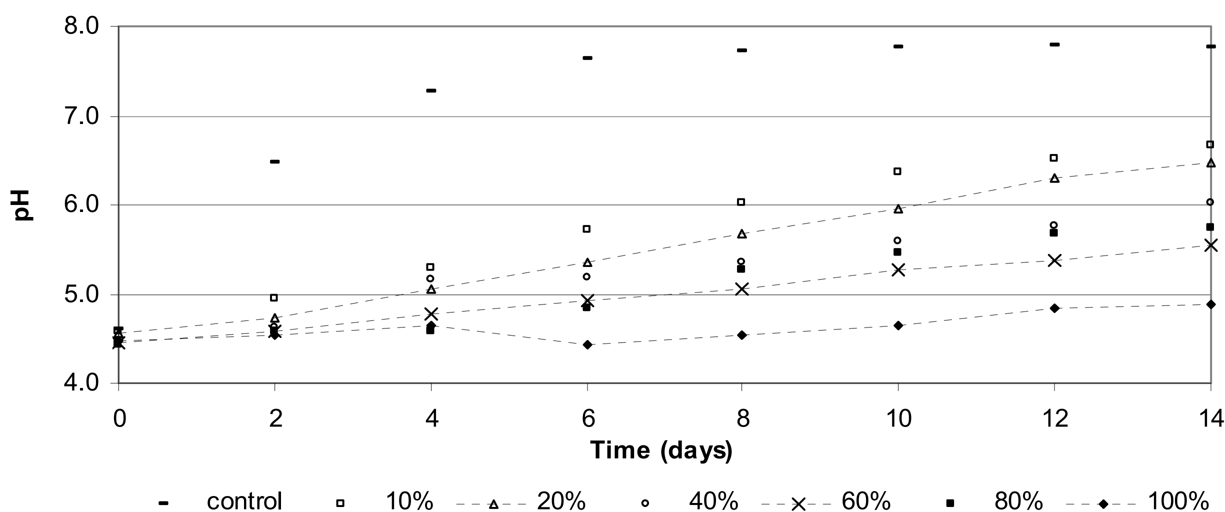


Figure 6.5: Change in pH over a 14 day period for various Amarula wastewater concentrations inoculated with *Trametes pubescens*. Highest standard deviation: 0.21 pH units.

An anomaly occurred with the 80 % wastewater concentration pH increase. Although the pH increase after fungal treatment was smaller as the wastewater concentration increased, the final pH at 80 % concentration was higher than that of the 60 % wastewater concentration. The 80 % concentration did allow for the highest laccase synthesis and it was the lowest wastewater concentration in which fungal growth and initial COD removal were shown to be inhibited (Figure 6.1). A decrease in pH was observed with the 100 % wastewater samples on day six. This was in accordance with prior work with brandy distillery wastewater (Chapter 4), which had observed a drop in pH during the exponential growth phase until the exhaustion of the carbon source. Although the pH had been increased during the experiment it would still require further modification should a further biological treatment step such as methanogenic fermentation be included in the treatment process, as anaerobic digestion occurs optimally between a pH of 7.0 and 7.5.

6.4.3 Laccase synthesis

A combination of factors including constituents in the growth medium such as the carbon source, nitrogen source, compounds and trace elements contribute to fungal growth. The concentrations of these constituents and inducer phenolic compounds are vital to laccase synthesis. Low concentrations of an easily utilisable carbon source and a lack of trace elements could all negatively affect laccase synthesis. Although the presence of some phenolic compounds can greatly improve laccase synthesis, the effects of an inducer vary greatly amongst fungal genera and the inducer concentration is vital. If present in excess, growth inhibition and subsequently low laccase synthesis occurs (Bollag and Leonowicz, 1984), while if present in too low a concentration no effect is observed.

The highest laccase activity in all samples at or below a 60 % wastewater concentration was measured within the first six days of sampling (Figure 6.6) and increased as the concentration of wastewater increased. The peak of highest laccase activity occurred with the removal of more than 60 % of the COD. The same trend was observed in the 100 % wastewater, except the laccase activity was lower than that obtained in the 60 % wastewater concentration and there was a lag phase of four days before laccase activity increased substantially. Laccase activity increased rapidly from the fourth to eighth days, after which it decreased slightly. The

low laccase levels over the first four days were attributable to the growth inhibition, which was probably due to the high concentration of phenolic compounds, as growth was visibly slower in the 100 % wastewater concentration than in all other wastewater concentrations.

Prior work by Galhaup *et al*. (2002b) observed an initial decrease in pH after inoculation using the same fungus in a batch culture, in which a laccase synthesis increase coincided with a pH increase. In contrast the pH decrease only occurred on day six in this study, two days after greater laccase synthesis had begun. The reason for this variation could be ascribed to the difference in the components of the synthetic medium used by Galhaup *et al*., (2002b) and the constituents of the Amarula wastewater. The synthetic medium had a high concentration of glucose as a carbon source, while the wastewater had a complex mixture of carbon-containing compounds extracted from the marula pulp. Various organic acids in the wastewater may have been utilised early as carbon sources and their degradation would have led to an increase in pH.

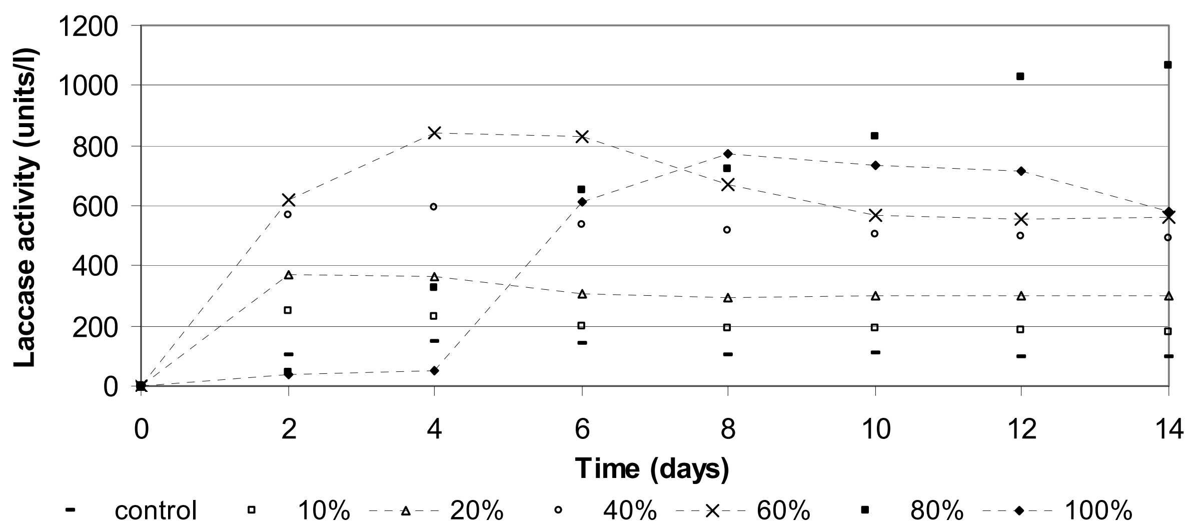


Figure 6.6: Laccase activity over a 14 day period for various Amarula wastewater concentrations inoculated with *Trametes pubescens*. Highest standard deviation: 130 units/l.

The large concentration of phenolic compounds in the wastewater inhibited growth, which may have led to a subsequent shift in metabolism to aid survival and acclimatisation. By the time increased carbon consumption and growth could occur, a greater proportion of the easily utilisable carbon sources in the wastewater would have been consumed, affecting the manner in which laccase would have been produced. Growth was shown to be inhibited in the 100 %

wastewater concentration as was evident in the lower initial COD removal. Laccase synthesis in the 80 % wastewater concentration did not conform to the trend of peaking and then decreasing. Instead laccase synthesis in the 80 % wastewater increased throughout the experiment and the increase tapered off over the last two days. It also resulted in the highest laccase activity (1063 ± 26 units/l) obtained in this study.

Enzyme production was considerably lower than that attained with the remediation of brandy distillery wastewater, where laccase activity in flask cultures peaked at 4644 ± 228 units/l (Chapter 4). Laccase synthesis in *T. pubescens* is known to be induced by the presence of copper (Galhaup *et al*., 2002a; 2002b) and atomic absorption spectroscopy revealed 0.11 mg/l of copper in the wastewater, which would have sufficed for laccase synthesis. These results are in between those obtained using other agricultural waste residues to produce laccase using this fungal strain. Osma *et al*. (2007b) had obtained laccase synthesis of 1570 units/l when using banana skins as a support substrate and 400 units/l using mandarin peels as a carbon source with a soy oil supplement (Osma *et al*., 2007a). This wastewater differed from any other tested in this study in that it had an extremely high concentration of suspended solids (10.5 g/l). This consisted entirely of lignocellulosic remnants of the marula pulp and may have negatively affected laccase synthesis. When a number of cellulosic and lignocellulosic supplements were tested in a synthetic solution not one benefited laccase synthesis (Chapter 8).

6.5 Interim summary and conclusions

Up to 77 % of the COD was removed from the Amarula wastewater by fungal treatment. There was a longer lag phase in COD removal at a 100 % wastewater concentration than occurred using lower wastewater concentrations. Maintenance of a high concentration of biomass in the wastewater would probably increase the COD removal rate tremendously and negate the long lag phase that would occur with a small inoculum. In order to obtain maximum enzyme production as well as an effective wastewater treatment, an ideal wastewater concentration would be 80 %, if using a low inoculum batch treatment.

Up to 92 % of the total phenolic compounds were removed by fungal treatment of the wastewater. The majority of the phenolic compounds were degraded within the first two days. The initial rapid decrease was enzymatic, while the remainder was fungal metabolic degradation and/or atmospheric oxidative degradation. This was a very high removal efficiency considering that the concentration of phenolic compounds was higher in the Amarula wastewater than any other distillery wastewater assessed in this study.

The only disadvantage to the fungal treatment was that the colour of the wastewater increased by 66 ± 17 % and 50 ± 12 % in 80 and 100 % wastewater concentrations. However, the change in colour was measured relative to the initial colour at pH 4.5. If the pH of untreated and treated solutions is adjusted to pH 7.0 using sodium carbonate powder there is a significant decrease in colour with the fungal treatment. When the pH was adjusted using a buffered solution, the change in colour was not as great as when adjusted with powder.

Maximum laccase activities increased with increasing wastewater concentrations, peaking at a concentration of 80 % (1063 ± 26 units/l after 14 days), and decreasing again at 100 % concentration to levels similar to the activity obtained at 60 %. The 80 % wastewater concentration was also the only concentration in which laccase activities did not reach a maximum and decrease again over time. For the inoculum size and conditions tested in this chapter the 80 % concentration was indicative of the optimal wastewater concentration that contained sufficient compounds required for growth and laccase synthesis or induction, while dilute enough to counter the negative effects of compounds that inhibited growth. Whether laccase could be purified in a financially viable step was beyond the scope of this study, but should the enzyme find application in the production of Amarula it could be added directly from the fungally-treated wastewater. However, it is unlikely to be competitive when wastewaters such as those from brandy distillation (Chapter 5) have yielded more than eight times higher laccase concentrations.

In summary this work has shown that it is possible to biologically treat Amarula wastewater, a wastewater that has a high concentration of phenolic compounds and COD, at full strength using the white-rot fungus *Trametes pubescens* MB 89. There was a substantial decrease in

potentially inhibitory phenolic compounds, which would potentially facilitate further biological treatment such as anaerobic digestion to produce energy in the form of methane. The fungal treatment resulted in the production of a valuable enzyme at concentrations of just over 1000 units/l.

Chapter 7

Characterisation of sixteen winery wastewaters, fungal remediation and subsequent methanogenic digestion

7.1 Introduction

The world wine production in 2000 was approximately 2.65 x 10^{10} l/year (Petruccioli *et al.*, 2000). The production of wine yields an equivalent, or even larger, amount of wastewater resulting from various washing operations during the crushing and pressing of grapes, as well as rinsing of fermentation tanks, barrels and other equipment or surfaces (Malandra *et al.*, 2003). These wastewaters have large seasonal fluctuations in volume and composition according to the wine produced and the period of production (vintage, racking and bottling). Winery wastewater typically has a COD of 0.8 to 12.8 g/l and an acidic pH between 3 and 4 (Petruccioli *et al.*, 2000). The COD can increase to 25 g/l depending on the harvest load and processing activities. Although wastewater that is discharged into the environment should have a pH of 5.5 to 7.5 and a COD below 75 mg/l (South African Water Act no. 36, 1998), winery wastewaters are often discarded with little, if any, treatment (Malandra *et al.*, 2003). Winery wastewater that mainly comes from the washing waters of equipment and bottles and from the cooling processes is generally non-toxic and not hazardous (Petruccioli *et al.*, 2002). However, certain cleaning and sanitising chemicals, such as chlorine and ammonia solvents, are toxic and hazardous to the environment and to biological wastewater treatment systems. Some wineries now use more benign cleaning and sanitising agents, such as hydrogen peroxide, ozone or hot steam, which have an additional advantage in that less rinsing water is required to remove the chemicals (Musee *et al.*, 2005).

Although waste minimisation is slowly being adopted in the wine industry its full potential has not been realised. Most of the waste minimisation attempts have been carried out in an *ad hoc* fashion and have proven to be inefficient in many cases. The primary reason for this inefficiency has been attributed to the lack of a systematic methodology of targeting specific waste streams (Musee *et al.*, 2005). An increasing trend with wineries in urban areas is the pretreatment of wastewater prior to channeling to local municipal wastewater treatment facilities. Generally, suspended solids are removed by filtration and sedimentation and the pH is elevated chemically to facilitate aerobic or anaerobic digestion. This decreases penalties incurred as a result of the acid pH and high COD. Rural wineries often dispose of their wastewater by irrigation as they have the luxury of space and low population densities to complain should foul odours arise.

Most municipal activated sludge plants located in wine production regions suffer a drastic increase in organic load during the grape harvest period, which often results in problems with biological treatment systems, such as decreased sludge settleability, sludge floc disintegration, increased suspended solids in treated wastewater and, in the worst case, complete process failure (Chudoba and Pujol, 1996). Brucculeri *et al*. (2005) studied co-treatment of municipal and winery wastewaters in a full scale conventional activated sludge process. The wastewater treatment plant obtained 90 % COD and 60 % nitrogen removal efficiencies for both an extended oxidation process during vintage (four months per year) and a predenitrification / oxidation process during the rest of the year. Good removal efficiencies have been observed with both aerobic and anaerobic biological treatment methods. Petruccioli *et al*. (2002) minimised energy consumption of aeration using a jet loop bioreactor containing activated sludge. Numerous versions of methods using anaerobic digestion are present in the literature. Torrijos and Moletta (1997) found a sequencing batch reactor to be the best option for the treatment of winery wastewater from smaller wineries and obtained greater than 90 % removal of total COD, soluble COD and biochemical oxygen demand. Continuous production of hydrogen from the anaerobic acidogenesis of a high-strength rice winery wastewater by a mixed bacterial flora in an upflow reactor has been demonstrated by Yu *et al*. (2002). Other forms of treatment include methods such as evaporation-condensation, ultrafiltration and reverse osmosis (Petruccioli *et al*., 2000).

Fungal treatment has the potential to remediate winery wastewaters and if laccase can be produced as a byproduct of the remediation, it too can be used in the wine-making process. Laccase has been proposed by Minussi *et al*. (2007) as an alternative to the physicochemical adsorbents that act on the polyphenols, which are responsible for the madeirisation process in wines. Traditional wine technology has used stabilising procedures such as proteinaceous clarification, use of polyamides and high doses of sulphur dioxide, which act on catalytic factors, block oxidisers or remove phenolic compounds. Although laccase has been shown to react with the phenolic compounds responsible for the antioxidant properties in red wine musts, it has shown a higher reduction of the total phenolic compounds than in the total antioxidant potential in white wine musts. Riesling wines that were stable and of high quality were made using a laccase treatment, showing that product instability could be prevented by

using enzymes instead of sulphur dioxide (Maier *et al.*, 1990). Enzymatic treatment has the potential to decrease costs associated with wine processing and improve wine stability over long storage times (Cantarelli and Giovanelli, 1990).

7.2 Objectives

The objectives of this study were to characterise wastewaters from a number of wineries during peak production and then investigate the use of white-rot fungi to lower the COD, the concentration of phenol compounds and colour, while increasing the pH to a level suitable for further treatment by anaerobic digestion. Laccase production during fungal remediation was also assessed as the wastewater could be utilised as a potential medium in which to produce the enzyme. Fungally-treated wastewater and untreated wastewater were then treated by methanogenic digestion to assess if a fungal pretreatment step was beneficial.

7.3 Materials and methods

Wastewater was obtained from fifteen wineries in the Western Cape Province of South Africa and stored at 4 ºC. Aliquots of 65 ml of unmodified undiluted wastewater were placed in 300 ml Erlenmeyer flasks, covered with aluminium foil (to prevent contamination) and sterilised by autoclaving for fifteen minutes. Duplicate samples were inoculated with *T. pubescens* MB 89 from stock cultures that had been cultured in a medium containing 2 % malt extract, 1 % glucose and 0.2 % yeast extract (all Merck, as above) and placed on a benchtop shaker (Labcon SP015+UPF75, Maraisburg) at 150 rpm at 28 ^{o}C. Control inocula in distilled water were conducted in duplicate. Samples of 1 ml were taken in 1.5 ml Eppendorf containers and centrifuged at 9660 *g* for two minutes (Heraeus Biofuge, Germany). The supernatant was aspirated, diluted appropriately and tested for laccase activity, COD concentration, total phenolic compounds concentration, pH, and colour. Samples that did not exhibit laccase synthesis were assayed for seven days, while samples that did were assayed for nine days. Laccase, COD, colour and total phenolic compounds and pH values were measured as in Chapter 3 (section 3.3.4).

Wastewater samples after fungal treatment were combined, centrifuged and 100 ml of the supernatants was placed in 100 ml Erlenmeyer flasks, as was 100 ml of the raw wastewaters.

The pH values were adjusted to between 7.0 and 7.5 and the samples were inoculated with a mixed culture of methanogens (kindly donated by Dr L. Dekker) while flushing with nitrogen gas to maintain anaerobic conditions. Distilled water served as the control flasks. The flasks were shaken at 50 rpm at 32 ± 2 ºC for a total of fourteen days. Samples of 1.5 ml were centrifuged at 9660 *g* for two minutes (Heraeus Biofuge, Germany). The supernatant was aspirated, diluted appropriately and tested for COD, total phenolic compounds concentration, pH and colour.

The concentrations of nitrite, nitrate, ammonia, phosphorus and chloride were all measured using Merck Spectroquant kits 1.14776, 1.14752, 1.14773, 1.4543 and 1.4828 respectively, which are the equivalent of standard methods 4500-NO_2^- B, 4500-NO_3^- E, 4500-NH_3 F, 4500-P C and 4500-Cl B respectively (APHA *et al*., 1998). Colour was measured at 525 nm and is presented as Hazen. Lactose fermenters were differentiated from non-lactose fermenters on spread plates using McConkey agar (Saarchem, Merck).

7.4 Results and discussion

7.4.1 Wastewater characterisation

The winery wastewater characteristics are shown in Table 7.1. The most significant characteristics that would hinder conventional treatment with anaerobic digestion were the pH (as acidic as 2.95), the presence of phenolic compounds (up to 95 mg/l) and COD values varying from 665 to 12600 mg/l.

Malandra *et al*. (2003) found wastewater composition of winery wastewater during the 1999 harvest season similar to this study, having a pH between 3.7 and 4.8, total polyphenols between 0 and 27.2 mg/l and a COD between 320 and 5670 mg/l. The results found in this study also coincide with those of Malandra *et al*. (2003) in that a large variation in COD values (and all other parameters tested) was observed. The considerable variation in wastewater constituents can be ascribed to different varieties of grapes, harvest load and operation procedures. The variation in wastewater composition is best illustrated in samples Q and F2, which represent two samples from the same winery a day apart. The COD values are more than ten times higher the second day and the pH varies from 2.91 to 4.80. The COD

values and total phenolic compounds concentrations were generally higher in this study than the one conducted by Malandra *et al*. (2003), even though both studies occurred during peak harvest season. This is possibly due to the variations in wastewaters and sampling procedures, but could also well reflect the change in practices since 1999. Less wasteful practices regarding water utilisation may be leading to lower volumes of more concentrated wastewaters. Similar variations in results were found by Bustamante *et al*. (2005), who measured various characteristics of 21 winery and distillery wastewaters. Some of their values were extremely high. They found the COD of the winery wastewaters to range from 296 to 738 g/l. The high COD value is possibly a direct sample of a wine lees, as a similarly high value of 210 g/l was obtained when characterising a lees in this study (see Table 5.1). Bustamante *et al*. (2005) found the pH values to range from 3.6 to 11.8, polyphenol concentrations from 29 to 474 mg/l, total nitrogen of 0.0 to 142.8 mg/l (35.4 mg/l mean) and phosphorus 3.3 to 188.3 mg/l (35.4 mg/l mean). The values for the phosphates measured in this study overlapped with the lower end of the range measured by Bustamante *et al*. (2005) for phosphorus. This was due to their measurement comprising total, acid-soluble phosphorus (which had a 0.98 correlation to total solids), while in the present study only dissolved phosphate was measured after all particulate matter had been removed by centrifugation.

Distillery wastewaters during 1999 were also characterised by Malandra *et al*. (2004) and they also found large variations in composition. The wastewater concentrations of total nitrates ranged from 35 to 132 mg/l, chlorides from 20 to 425 mg/l, phosphates from 98 to 251 mg/l and ammonia levels ranged from 68 to 378 mg/l. The values in the present study were generally below these values. This would be expected as distillery wastewaters are generally high strength wastewaters that have not been exposed to the environment and various microorganisms. Many of the winery wastewater samples could only be collected from holding tanks, where a fair amount of degradation may already have occurred. Lactose, non-lactose fermenters or both were present in all samples. The dominance of certain microorganisms varies over time. Jourjon *et al*. (2005) observed that acetic and lactic acid bacteria and yeasts were dominant in winery wastewater treatment systems at the beginning of the harvest. The presence of these microorganisms progressively diminishes during the year until the aerobic microbes become dominant.

Table 7.1: Characteristics of winery wastewaters. Total phenols, COD, NH_3, NO_2^-, NO_3^- and Cl^- (mg/l) and colour (Hazen). A single 25 L sample was collected and this was tested in triplicate.

Winery	pH	Total phenols	COD	Colour at 525 nm	PO_4^{3-}	NH_3	NO_2^-	NO_3^-	Cl^-	Lactose fermenters	Non-lactose fermenters
A	3.68	85	12467	20	3.59	0.04	0.28	5. 6	203	+	+
B	4.11	95	12600	13	4.28	0	0.02	20.4	70	+	+
C	3.68	33	11600	6	4.1	0.03	0.00	17.1	170	-	-
D	4.22	56	10067	2	4.85	0.38	0.15	14.7	107	+	+
E*	**4.81**	**27**	**9533**	**4**	**2.1**	**0.03**	**0.06**	**56.2**	**76**	**+**	**-**
F	4.80	7	7167	5	0.01	0.00	0.02	1.4	124	-	+ very low
G*	**3.92**	**54**	**6217**	**10**	**4.45**	**0.00**	**0.03**	**0.0**	**203**	**-**	**+**
H	3.96	38	5767	10	5.04	0.05	0.03	3.1	162	+	-
I	4.44	19	4583	3	2.59	0.02	0.23	39.5	203	+	+
J*	**4.15**	**32**	**4100**	**3**	**1.35**	**0.05**	**0.03**	**2.2**	**101**	**+**	**+**
K*	**5.05**	**27**	**3300**	**1**	**3.88**	**0.04**	**0.11**	**6.9**	**39**	**+**	**+**
L	4.55	16	2700	1	1.41	0.03	0.04	5.1	136	+	+
M*	**4.15**	**29**	**2300**	**8**	**2.32**	**0.01**	**0.01**	**82**	**17**	**+**	**+**
N	5.03	15	1767	3	2.15	0.10	0.00	12.7	144	+	+
O	4.04	6	1267	1	0.00	0.11	0.06	0.0	151	+	-
Q	2.91	5	667	3	4.69	2.69	0.00	0.0	126	-	+ very low
Mean	4.22	34	6006	6	2.9	0.22	0.07	131.1	13		

*Samples that produced laccase during fungal treatment.

7.4.2 Chemical oxygen demand

The initial COD values of the sixteen samples varied from 665 to 12600 mg/l, with a mean of 6020 mg/l. The COD values of all the wastewater samples were reduced by the fungal treatment (Figure 7.1). The average removal efficiency in the eight samples with the highest COD values (i.e. samples A to H) was 84 %, while the average removal in the eight samples with the lowest COD values (i.e. samples I to Q) was 45 %.

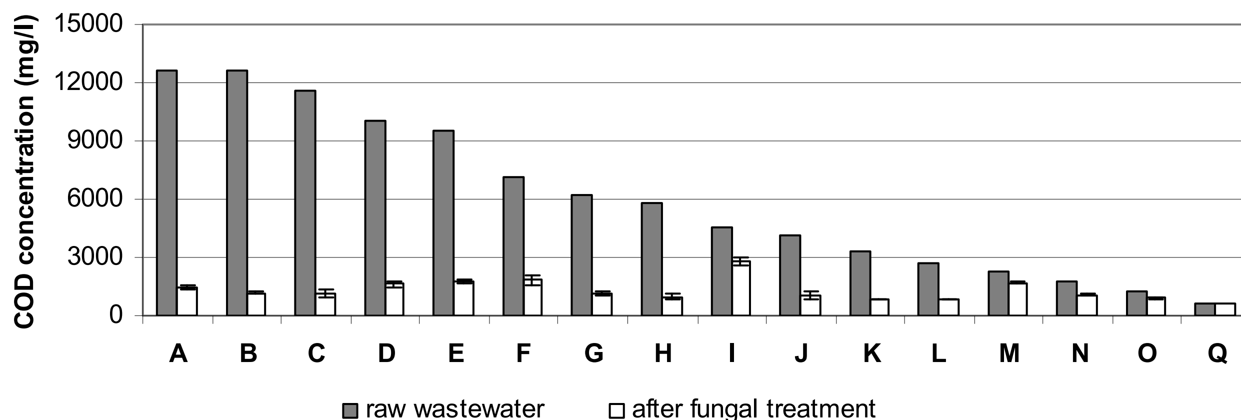


Figure 7.1: Change in COD after aerobic fungal digestion (after 7 or 9 days for E, G, J, K and M) of undiluted winery wastewaters. Error bars represent standard deviation.

Petruccioli *et al*. (2002) obtained a slightly higher overall COD efficiency (above 90 %) using a jet-loop activated sludge reactor over a period of 12 months. The wastewater COD values were between 8000 and 12800 mg/l, with COD organic loading rates varied between 0.4 and 5.9 kg COD/m^3/d and a hydraulic retention time between 2.1 and 4.4 days. The decrease in COD was comparable (87 ± 4 %) for the five samples having initial COD values greater than 9000 mg/l. However, the time taken for this digestion in shake-flask cultures was substantially longer (seven days, for samples not producing laccase, or nine days for samples that were producing laccase). Increasing the biomass concentration (initially at 0.85 g dry mass/l) and providing aeration would reduce this time considerably for the same level of conversion, as greater metabolism would occur with the increased mass transfer and oxygenation. Duarte *et al*. (1997) observed an increase in aeration improved COD removal. However, aeration costs are often expensive, so the benefits must appropriately offset the costs of the procedure. Fungal pretreatment does have advantages in that it is often able to treat wastewaters that are toxic to many microorganisms, and value-added products such as proteins and enzymes may be produced. Jiménez *et al*. (2006) showed that the total solids, volatile solids, total suspended solids, volatile suspended solids and total volatile fatty acids

(acetic acid) were more than halved with fungal pretreatment of beet molasses alcoholic fermentation wastewater.

The COD concentrations of both raw and fungally-treated wastewater samples were reduced by treatment with methanogens (Figure 7.2). Keyser *et al*. (2003), evaluating upflow anaerobic sludge blankets for the treatment of winery wastewater, obtained 90 % and 85 % COD removal efficiencies with 24 hour hydraulic retention time with reactors seeded with granular sludge enriched with *Enterobacter sakazakii* or with brewery granules, respectively. However, they also observed that a reactor seeded with just sludge showed typical problems encountered with conventional sludge seeding and had to be re-seeded continuously, as with the flasks in this study. The flasks had to be reseeded with methanogens after five days, as very little digestion had occurred.

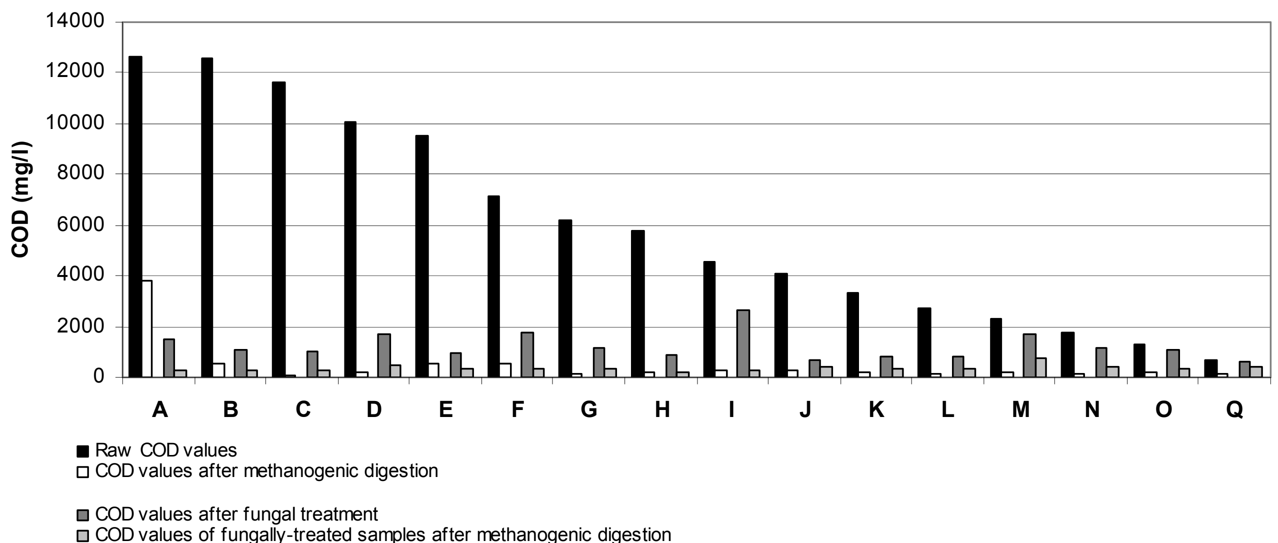


Figure 7.2: Decrease in COD with methanogenic digestion of fungally-treated and raw wastewater after 14 days.

Pretreatment with fungi prior to methanogenic digestion appeared to offer no distinct advantage with respect to the post-digestion COD concentrations. Although one wastewater sample was more resistant than the other wastewaters to methanogenic treatment after fungal treatment, there was generally no difference in final COD between fungally-treated and raw samples. Only the winery samples A and M raw were not degraded to a final COD concentration below 600 mg/l. By the end of the digestion the average reduction in COD concentration was 484 ± 905 mg/l for the untreated samples and 359 ± 118 mg/l for the fungally-treated samples. If the values for winery wastewater A were omitted, the average COD concentration after methanogenic digestion was 261 ± 157 mg/l for the untreated samples and 366 ± 119 mg/l for the fungally-treated samples. It appeared that the fungal

treatment slightly elevated the concentration of compounds recalcitrant to methanogenic fermentation.

There is a great increase in wastewater volume during the harvest and grape-processing season. Shorter treatment times are a great advantage when peaks occur in wastewater volume. Jiménez *et al*. (2006) reported that the pretreatment of vinasses with *Penicillium decumbens* increased the kinetic constant for subsequent anaerobic digestion process 6.9 times. They attributed this and the reduction in toxicity to the decrease in total phenolic compounds (0.45 to 0.145 g/l). From the parameters they tested it does, however, seem likely that some other component of the COD (80.5 to 23 g/l) may also have contributed to the toxicity of the wastewater, as high inorganic salt concentrations have also been implicated in decreasing biological degradative efficiency. Often real wastewaters prove more resistant to treatment than synthetic solutions and conditions. A yeast that was able to completely degrade relatively high concentrations of caffeic acid, vanillic acid, p-coumaric acid, gallic acid and catechol was found to be ineffective when treating olive mill wastewater (Passarinho *et al*., 1998). Mosteo *et al*. (2006) showed that winery wastewaters could be degraded by using the photo-Fenton process in heterogeneous phase. Maximum total organic carbon removal of 55 % was achieved for synthetic samples that were prepared by diluting commercial grape juice or red wine in water. The time period of the photo-chemical reaction was not given though.

The data obtained in this study compare favourably to previous data presented by Mulidzi (2006), who used a constructed wetland to treat winery and low strength distillery waste. An average annual COD removal of 80 to 83 % was obtained with a hydraulic retention time of fourteen days. Although the hydraulic retention time was fairly long, constructed wetlands do offer the advantage of being a relatively low-maintenance technology once established, and therefore low cost if the land is available. The treated wastewater in Mulidzi's (2006) study was also shown suitable for use for irrigation and used to produce a cash crop in the form of cabbages.

7.4.3 Change in pH

The average initial pH value of the sixteen samples was 4.22, with values varying from 2.91 to 5.05. The pH values of all the raw samples increased as a result of the fungal treatment (Table 7.2). The average pH increase in the eight samples with the highest COD values (samples A to H) was 1.87 pH units, while the average final pH in the eight samples with the

lowest COD values (samples I to Q) was 2.84 pH units. Even though less metabolic activity occurred in the samples with the lower initial COD values, the greater change in pH was probably due to a lowered buffering effect.

Table 7.2: pH values of wastewaters before and after fungal digestion (after 7 or 9 days for E, G, J, K and M) and methanogenic digestion (after 14 days).

Winery wastewater sample	pH values after methanogenic digestion	pH values after fungal treatment	pH values of fungally-treated samples after methanogenic digestion
A	7.71	7.4	8.05
B	8.07	7.38	7.81
C	8.66	6.67	8.21
D	8.28	6.73	8.34
E	8.34	7.84	8.3
F	8.76	7.56	7.82
G	8.44	7.33	7.74
H	8.87	7.31	7.87
I	8.11	6.65	7.84
J	8.03	7.49	7.88
K	7.77	7.22	8.43
L	7.75	7.43	8.2
M	8.66	7.25	7.63
N	8.39	7.13	7.93
O	6.18	7.18	8.4
Q	7.83	7.65	8.38

The pH values of all the fungally-treated and raw wastewater samples were adjusted to between 7.0 and 7.5 with sodium carbonate in order to facilitate methanogenic digestion. The pH values of all the samples were adjusted daily over the first seven days of methanogenic digestion using sodium carbonate powder if they fell below 6.5. The greatest daily decreases in pH occurred in the raw wastewater samples that had higher initial COD values, possibly as a function of greater metabolic activity. By the end of the digestion the average pH values were very similar for the raw wastewater samples (8.12 ± 0.6) and for the fungally-treated wastewater samples (8.05 ± 0.27). Duarte *et al*. (1998) also observed the pH to increase with increasing digestion time and attributed that to the metabolic degradation of various acids present in the wastewater.

7.4.4 Degradation of phenolic compounds

The greatest removal efficiencies of phenolic compounds were obtained with samples that had the highest initial total phenolic compounds concentrations (Figure 7.3). The total phenolic compounds concentration for all but three of the sixteen samples decreased to its lowest value after two days of fungal treatment. The slight increase in phenolics concentration by the end of the fungal treatment indicated that a phenolic compound was synthesised during fungal

treatment, as had occurred with the treatment of distillery wastewaters. Although the phenol synthesis was low, it made a substantial percentage increase in wastewater samples that originally had very low phenolic concentrations. Laccase degradation of catechins has previously been shown to be very fast, while degradation for stilbenes (*cis* and *trans* resveratrol) and derivatives of cinnamic acids (ferulic and caffeic acid) and benzoic acids (syringic, vanillic and gallic acid) is relatively slow (Minussi *et al.*, 2007). Although laccase and fungal metabolism may have degraded phenolic compounds present in the wastewater, the fungi themselves may have released phenolic compounds as a result of metabolic activities or with cell autolysis.

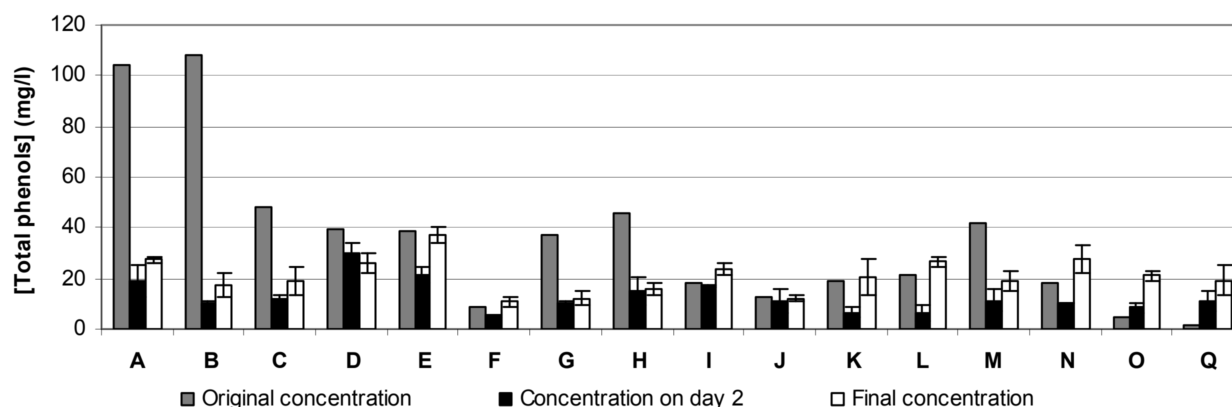


Figure 7.3: Change in total phenolic compounds concentration after aerobic fungal digestion (final concentration after 7 or 9 days for E, G, J, K and M) of undiluted winery wastewaters. Error bars represent standard deviation.

Anaerobic digestion removed phenolic compounds from both the raw wastewater and fungally-treated wastewater. The total removal was less than had occurred during fungal treatment. When comparing the data that were used to construct Figure 7.3 and Figure 7.4 it was evident that the laccase-producing fungi reduced the total phenol levels more in two days than anaerobic digestion did in two weeks for most of the samples. However, it was notable that six of the fungally-treated samples had significant further decreases in phenolic compounds, indicating that the anaerobic digestion was capable of removing some phenolic compounds that the fungal treatment did not, or that were produced by the fungi themselves.

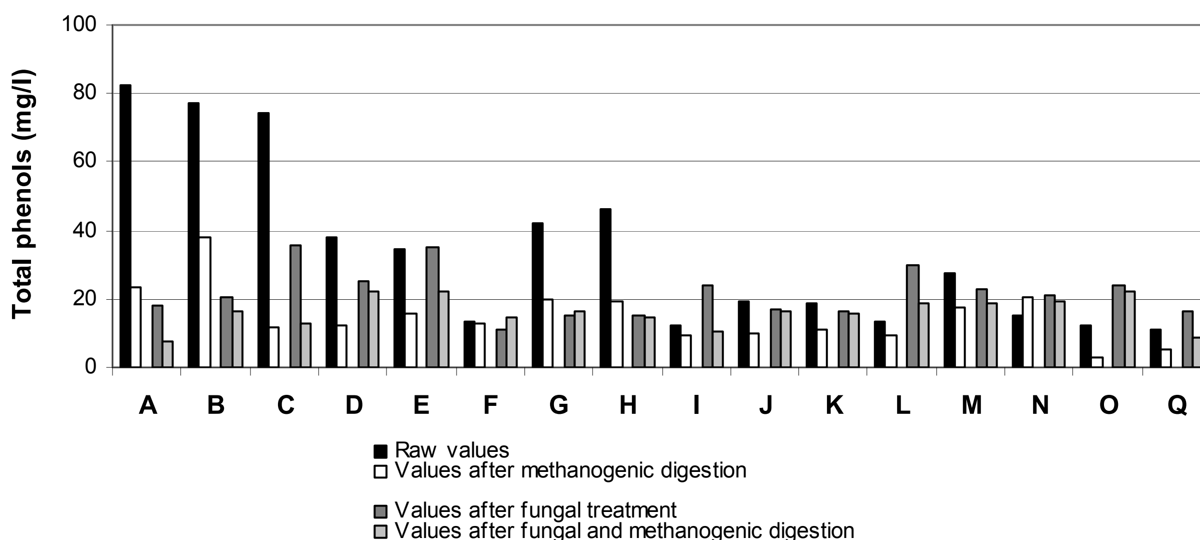


Figure 7.4: Change in concentrations of total phenolic compounds with methanogenic digestion of fungally-treated and raw wastewater.

7.4.5 Change in colour

In winery wastewaters various phenolic compounds found in the skin of the red grapes are responsible for the colour. The most distinctive is the red to purple colour that is imparted by the anthocyanins (glycosylated-anthocyanins). The samples with greater concentrations of phenolic compounds ranged in colour from light purple to light red. The colour decreased as a result of the fungal treatment in all but the three samples that had the lowest initial COD values (Figure 7.5). The decrease in colour correlated well to the decrease in total phenols. For the two samples with highest initial total phenolic compounds concentrations the decrease in colour was substantial.

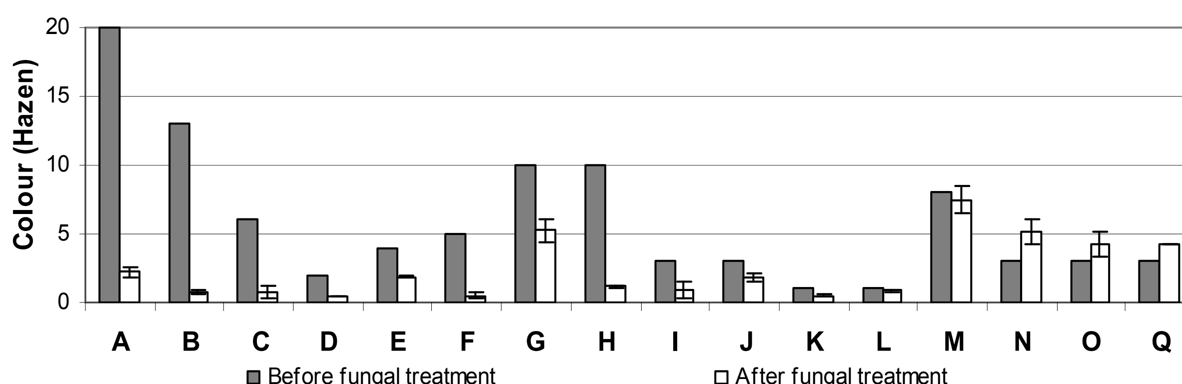
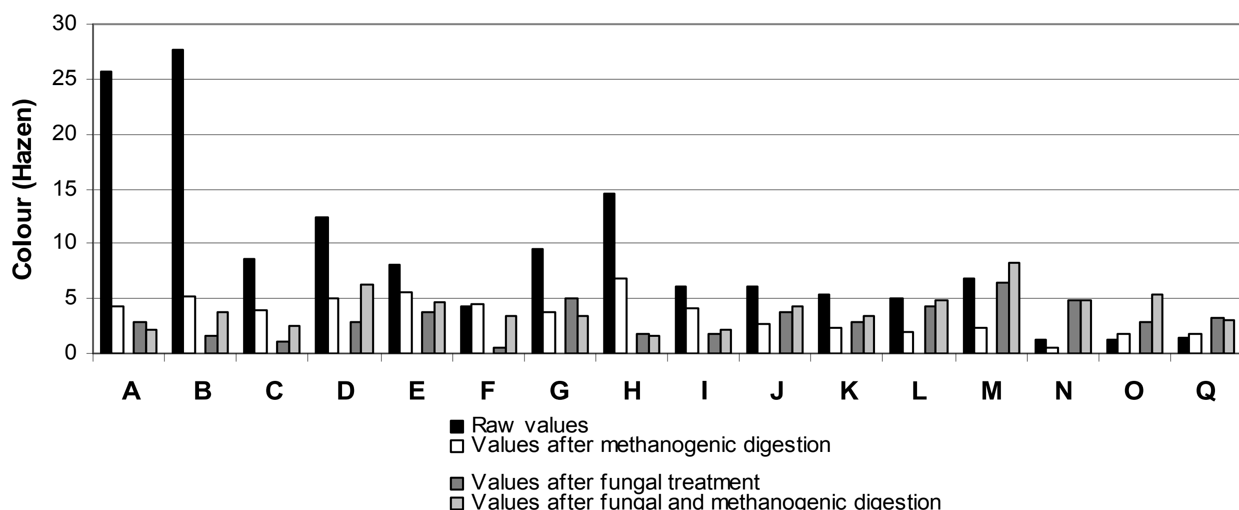


Figure 7.5: Change in colour after aerobic fungal digestion (after 7 or 9 days for E, G, J, K and M) of undiluted winery wastewaters. Error bars represent standard deviation.

Fungal remediation of distillery wastewaters originating from the distillation of wine showed *T. pubescens* capable of degrading the colour-containing compounds such that the wastewater was changed from a dark red/purple colour to a light yellow (Chapter 5).

The anaerobic digestion decreased the colour markedly for all but two of the sixteen raw samples (Figure 7.6). The fungally-treated samples all had much lower initial colour absorbance values, which led to less significant decreases being obtained. A recent review by Pant and Adholeya (2007) states that alcoholic fermentation of molasses results in a wastewater with a large concentration of a brown compound. The brown colour in the molasses vinasse results from phenolic compounds (tannic and humic acids) originating from the feedstock, melanoidins that result from Maillard reaction between sugars (carbohydrates) and proteins (amino groups), caramels from overheated sugars, and furfurals from acid hydrolysis. Conventional treatments reduce the colour in these wastewaters minimally and the colour may even be increased during anaerobic treatment (due to repolymerisation of compounds). The colour increased in a number of the samples after anaerobic treatment of the fungally-pretreated wastewaters. It is possible that some of the compounds that were depolymerised by the fungal treatment were repolymerised during the anaerobic treatment. Most of the samples displayed a slight increase in colour during the digestion, possibly due to



the increase in pH.

Figure 7.6: Change in colour with methanogenic digestion of fungally-treated and raw wastewaters after 14 days.

It has been observed that in wines the oxidation of certain compounds resulted in an increase in colour. Somers and Evans (1986) observed that atmospheric oxidation was not necessary for ageing reactions to occur in high and low phenol wines, but in the presence of air the loss of monomeric anthocyanins and the increase in polymeric pigments and chemical ageing was more rapid. An early increase in colour density was noted with air contact, which gradually declined until colour density of aerobic and anaerobic wines was very similar. This means that

if these compounds were present in the wastewater the natural oxidation would result in a colour increase.

7.4.6 Laccase synthesis

Of the sixteen wastewater samples inoculated with *T. pubescens* only five displayed an increase in laccase synthesis. The increase could not be attributed to the initial COD or total phenol concentration. The laccase values attained were very low for this particular fungus, the highest being 384 units/l (see Figure 7.7). Treatment of a brandy distillery wastewater had previously yielded laccase concentrations of 4644 ± 228 units/l in shake-flask cultures (see Chapter 4).

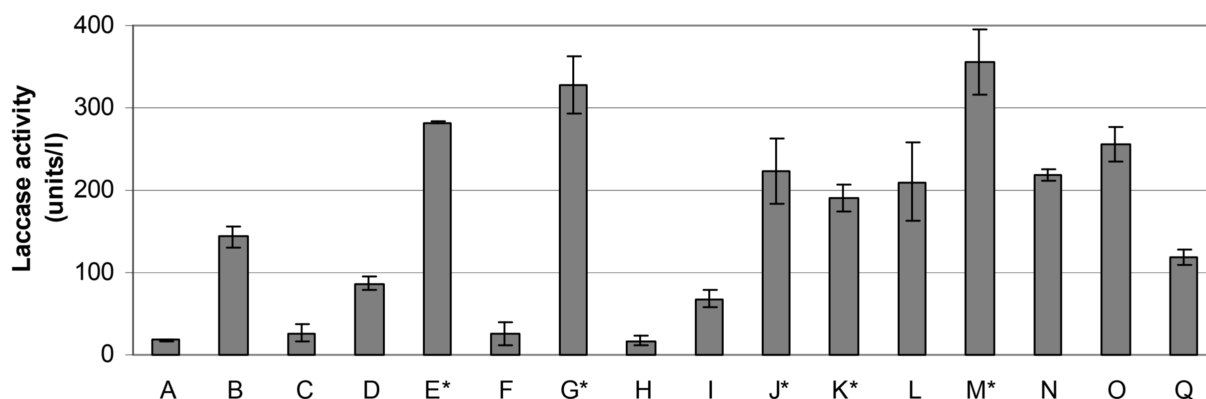


Figure 7.7: Highest laccase activities (after 7 or 9 days for E, G, J, K and M) resulting from the treatment of undiluted winery wastewaters. Error bars represent standard deviation and *indicates samples that increased in laccase activity.

The synthesis of laccase in submerged cultures of fungi has been shown to be dependent on factors such as the carbon source, nitrogen source, pH, the presence of inducers such as copper or phenolic compounds as well as sufficient agitation and oxygen supply. Copper is a vital component of laccase and may have been present in concentrations too low to allow for synthesis of the metalloenzyme. There is a large body of literature showing that various phenolic compounds stimulate the production of fungal enzymes. A number of these enzyme-inducing phenolic compounds are present in wine, notably red wines, and thus should be present in fractions of the cellar wastewaters (discussed in Chapter 2). The lack of laccase synthesis was probably attributable to two primary factors: an insufficient carbon source and an acidic pH. Visible biomass growth was marginal compared to cell concentrations attained in the inoculum's growth media. Malandra *et al*. (2003) have found that wastewater generated from the destemming and pressing operations contained relatively high concentrations of glucose, fructose and malic acid that originated from the grapes themselves. Unfortunately

dilution from rinsing and prior digestion from microorganisms already present may have removed a large portion of the easily utisable carbon source. Additionally wastewater from cellars generally contains a variety of other compounds as well as cleaning agents, which may hamper growth and laccase synthesis.

7.5 Interim summary and conclusions

Winery wastewaters were treatable by a pure culture of *Trametes pubescens* MB 89 with up to 91 % COD removal efficiency and 90 % removal of total phenolic compounds. The pH was increased in all samples after fungal treatment potentially making them amenable to anaerobic digestion. The colour and total phenolic compounds concentration decreased for wastewater samples having high initial values, while both characteristics increased for samples that had low initial values. Relatively low concentrations of laccase were produced even though a few of the samples had relatively high COD values for winery wastewaters. Laccase was synthesised in five of the sixteen samples, but at low concentrations that could not be related to the parameters assayed for in the characterisation studies.

There appeared to be little advantage in fungally pre-treating the wastewater as the same levels of degradation were obtained using anaerobic digestion of raw and pretreated samples. The additional costs in maintaining a monoculture of *T. pubescens* would not be justifiable as an initial treatment step. If phenolic compounds do inhibit biological treatment processes such as anaerobic digestion it may be that an aerobic pretreatment step with laccase and/or other enzymes may prove to be a more feasible alternative. However, no such limitation was observed with methanogenic digestion for the majority of the pH-adjusted, raw wastewaters in this study. Other factors that may inhibit anaerobic digestion include disinfectants, sanitisers and inorganic ions. Ideally these wastewater streams would be separated from the biological treatment stream to allow for optimal biological degradation. Low maintenance technology such as constructed wetlands followed by use for irrigation of a nonfood crop such as timber trees, or more intensive treatment systems such as methanogenic digestion, which generate a product in the form of biogas, appear to be more viable alternatives for winery wastewater treatment.

Chapter 8

Increasing laccase synthesis using a submerged culture of *Trametes pubescens* MB 89 in synthetic solution and modified wine-related wastewaters

8.1 Introduction

Numerous studies have investigated the most favourable conditions for laccase production by various fungi with solid and submerged fermentations (Gianfreda *et al*., 1999). The production of laccase is dependent on a number of factors, including the strain of fungi (or genetic manipulation thereof), the composition of the culture medium (compounds that provide a nitrogen and carbon source or that act as inducers), the cultivation method (stationary or agitated cultures) and the culture conditions (oxygen availability, pH of the medium, temperature of cultivation). In basidiomycete fungi, extracellular laccases are constitutively produced in small amounts (Bollag and Leonowicz, 1984). Some fungi, such as *Lentinus edodes*, produce high concentrations of laccase during primary growth, which is advantageous as this shortens the fermentation period required for maximal laccase production (Crestini *et al*., 1996). The majority of the literature shows laccase to be produced in appreciable concentrations during idiophase, when the presence of the inducer (or possibly its metabolite) in combination with a decrease in compounds (such as the more easily utilised carbon source) to below a critical concentration leads to enhanced laccase synthesis.

Laccase synthesis can be notably increased by the addition of certain compounds or ions known as inducers. Inducers are compounds that elicit an enhanced production of enzyme while occurring at concentrations that are extremely low relative to available carbon sources. Greater laccase synthesis is dependent upon the presence of an inducer, its concentration and time of addition (Gianfreda *et al*., 1999). Fungal genera differ markedly in their inducibility with various phenolic compounds, many of which are phenolic or aromatic compounds related to lignin or lignin derivatives. The inductive effect has been found to be dependent on very small differences in molecular structure, since there are large differences between the inducer effects of compounds that are very similar (Shuttleworth and Bollag, 1986). Non-phenolic compounds such as ethanol (Alves *et al*., 2004) and metal ions such as copper (Galhaup *et al*., 2002a; 2002b) have also been shown to increase the levels of laccase synthesis; however, ethanol has often been used in concentrations that would constitute it a carbon source more than a classical inducer. The inducer concentration is integral to increasing laccase synthesis. Too low a concentration and no effect is observed, too high and a toxic effect is often observed in the form of growth inhibition. There are problems associated with use of these aromatic compounds for laccase production. They are often toxic to humans and can be expensive. The identification of environmentally sound and

economically feasible compounds that stimulate laccase production is still an ongoing concern.

A variety of agro-industrial waste residues may be utilised to produce laccase and thereby lower the substrate costs involved in production. The value of the enzyme produced in a wastewater treatment process may also allow for a treatment process that would ordinarily not be affordable, as the product generated can offset the associated process or capital costs. Barley bran (Rodríguez Couto *et al*., 2002), a common waste from the brewing industry, chestnut shell waste from glacé chestnut production (Gomez *et al*., 2005), banana skins (Osma *et al*., 2007b), mandarin peels (Osma *et al*., 2007a), kiwi fruit wastes (Rosales *et al*., 2005) and grape seeds (Rodríguez Couto *et al*., 2006) have all been utilised in order to produce laccase using white-rot fungi. Minor adjustment or supplements could greatly improve the concentration of laccase attained when utilising these wastes as a substrates.

8.2 Objectives

The objective of this chapter was to determine how to enhance laccase synthesis by *T. pubescens* MB 89 with pH adjustment, carbon supplementation and the addition of a variety of inducers. The most stimulatory inducer was to be assessed further using different numbers of additions, at different times to determine which would have the greatest positive impact on enzyme synthesis. These modifications or supplementations that were most beneficial to laccase synthesis were to be assessed in wine-related wastewaters to establish whether laccase synthesis could be consistently enhanced.

8.3 Materials and methods

8.3.1 The effect of pH on laccase synthesis, chemical oxygen demand removal and total phenolic compounds removal from wastewater

The pH of a brandy distillery wastewater (COD 29.5 g/l, total phenolic compounds 280 mg/l and pH 3.75) was adjusted to 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0 using hydrochloric acid or Na_2CO_3 powder (both Saarchem, uniLAB, Merck). Aliquots of 65 ml of the wastewater were placed in 250 ml Erlenmeyer flasks, covered with aluminium foil (to prevent contamination) and autoclaved for fifteen minutes. Duplicate flasks were inoculated with *T. pubescens* MB 89 (0.87 ± 0.28 g/l) from stock cultures that had been grown in liquid cultures containing 2 % malt extract, 1 % glucose and 0.2 % yeast extract (all Merck, as in section 3.3.1). Laccase

activity, total phenolic compounds concentration and COD were measured using methods described in section 3.3.4.

8.3.2 The effect of different carbon, nitrogen, lignin/cellulose sources and phosphorus

Different carbon sources in the form of fructose, glucose, mannitol, maltose, sucrose, cellobiose and lactose (all Saarchem, univAR, Merck) were supplemented into a brandy distillery wastewater (COD10.5 g/l, total phenolic compounds 35 mg/l and pH 3.9) to assess their individual effects on laccase synthesis. The amount added was the equivalent to the molar equivalent of carbon atoms in 10 g/l of glucose. Different nitrogen sources in the form of NH_4NO_3, NH_4Cl, KNO_3 (Saarchem, univAR, Merck) and H_2NCNH_2 (analaR, BDH) were added at a molar equivalent of nitrogen atoms in 2 g/l of KNO_3, while malt extract, yeast extract and peptone were added at 2 g/l. Cellulose and lignin-containing additives in the form of cellulose powder, bluegum powder, rooibos tea leaves (*Aspalathus linearus*) and sugarcane bagasse were supplemented at 1 g/l and a phosphorus source (H_3PO_4) was also supplemented into the low strength wastewater. In all cases the wastewater pH was adjusted to 5.0 using sodium carbonate powder. Aliquots of 65 ml of the solutions were placed in 250 ml Erlenmeyer flasks, covered with aluminium foil (to prevent contamination) and autoclaved for fifteen minutes. Triplicate flasks were inoculated with *T. pubescens* MB 89 (1.27 ± 0.31g/l) from the stock cultures described above. The flasks were placed in a shaking incubator (Labcon) at 150 rpm at 28 °C for 15 days. Control flasks were inoculated and cultured in the media containing no inducers. Samples were taken from the flasks at least every second day, centrifuged in 1.5 ml Eppendorf containers at 9660 *g* for two minutes (Heraeus Biofuge, Germany) and the supernatant was diluted appropriately and tested for laccase activity.

8.3.3 The effect of inducers

8.3.3.1 Addition prior to or four days after inoculation

Inducers in the form of 3, 4-dimethoxybenzyl alcohol, 2, 5-dimethylaniline (2, 5-xylidine), syringic acid, violuric acid, hydroxybenzotriazole (HBT) (all Fluka, Sigma Aldrich Ltd, Cape Town), guaiacol, *p*-coumaric acid, 2, 6-dichloroindophenol, quercetin dehydrate, *o*-cresol, gallic acid (DI) (all Sigma), *n*- hydroxypthalamide, 4-methylcatechol (both Aldrich), phenol, phenol red and copper (all Saarchem, uniLAB, Merck) were all tested at 1 mM, while cycloheximide (Sigma-Aldrich), an antibiotic, was tested at 0.1 mM. Tannic acid (Sigma), cellulose powder (Aldrich, 20 micron diameter), rooibos tea leaves and absolute ethanol (Merck) were tested at 0.1 % (w/v) in 250 ml Erlenmeyer flasks containing 65 ml of a

synthetic medium comprising: glucose (Saarchem, uniLAB, Merck) (20 g/l), peptone (3 g/l), malt extract (both Biolab, Merck) (3 g/l), KH_2PO_4 (1 g/l), $Na_2HPO_4.2\ H_2O$ (100 mg/l), $MgSO_4.7\ H_2O$ (500 mg/l), $CaCl_2$ (10 mg/l), $FeSO_4.7\ H_2O$ (10 mg/l), $MnSO_4.4H_2O$ (1 mg/l), $ZnSO_4.7\ H_2O$ (1 mg/l) and $CuSO_4.5\ H_2O$ (2 mg/l) (all Saarchem, uniLAB, Merck). All inducers other than ethanol were autoclaved in the synthetic medium (pH adjusted to 5.0 individually after the addition of the inducer). Absolute ethanol was autoclaved separately and added just prior to the addition of the inoculum. To test the effects of inducer addition, after five days autoclaved flasks containing only the synthetic medium were inoculated. The solid inducers were weighed individually, autoclaved in Eppendorf tubes and added aseptically after five days of growth. Liquid inducers were autoclaved in glass McCartney bottles and aseptically pipetted into the flasks after the same time interval. All inducers and controls were performed in triplicate at 28 °C on a shaking platform (Labcon) at 150 rpm for 20 days. Samples of <0.5 ml were taken daily for 2, 5-xylidine for the first six days after addition, otherwise samples were taken every 48 hours.

8.3.3.2 Multiple additions of 2, 5-xylidine

One inducer, 2, 5-xylidine, was additionally tested in the synthetic medium by varying the time and number of additions. The inducer was autoclaved and added aseptically such that the concentration was increased by 1 mM with each addition. One or two additional doses were given two days apart, commencing at different times after inoculation (see Table 8.4 in this chapter for the times and numbers of addition).

8.3.4 Laccase synthesis in supplemented distillery wastewaters and a synthetic wine lees

Samples of four wastewaters were obtained from a winery and two distilleries near Worcester in the Western Cape Province of South Africa and stored at 4 ºC. Two of the wastewaters (a wine lees and a distilled wine lees which had had its tartaric acid extracted) were tested at 30 % concentration, while two distillery wastewaters were tested at full strength. The pH of all four wastewaters was adjusted to 5.0 using Na_2CO_3 powder (Saarchem, uniLAB, Merck). Aliquots of 65 ml were placed in 250 ml Erlenmeyer flasks, covered with aluminium foil (to prevent contamination) and sterilised by autoclaving for fifteen minutes. Triplicate flasks of all wastewaters were inoculated with biomass of *Trametes pubescens* MB 89 (0.76 ± 0.25 g/l) from the stock cultures described above. Control inocula were conducted in the raw, unmodified wastewaters. The flasks containing the wastewater samples were placed on a bench top shaker (Labcon SP015+UPF75, Maraisburg) at 150 rpm at 28 °C for 14 days.

Laccase activities were tested every 48 hours for all flasks for two weeks except for 2, 5-xylidine, which was tested every 24 hours for the first eight days and every 48 hours thereafter.

8.4 Results and discussion

8.4.1 The effect of pH on laccase synthesis, COD removal and total phenolic compounds removal

Laccase synthesis was sensitive to the pH range that was tested in the brandy distillery wastewater. A clear advantage was seen using pH 5.0, as synthesis at a pH only 0.5 units more acidic and basic was over 40 % lower (Figure 8.1). At more acidic pH values there may have been a longer lag phase in growth, which resulted in much of the nutrients being consumed and thereby decreased potential nutrients that the cells could store prior to reaching stationary phase. This could have shortened the time period during the stationary phase during which laccase could be synthesised. It was observed in Chapter 6 that growth inhibition had lowered the concentration of laccase synthesised in Amarula wastewater. At higher pH values there may be no requirement to synthesise an enzyme such as laccase, which is relatively ineffective in comparison to activities under more acidic conditions below pH 4.5. The optimal range for the laccase isoforms secreted by this fungal strain is between pH 3.0 and 4.5 (Galhaup *et al*., 2002a).

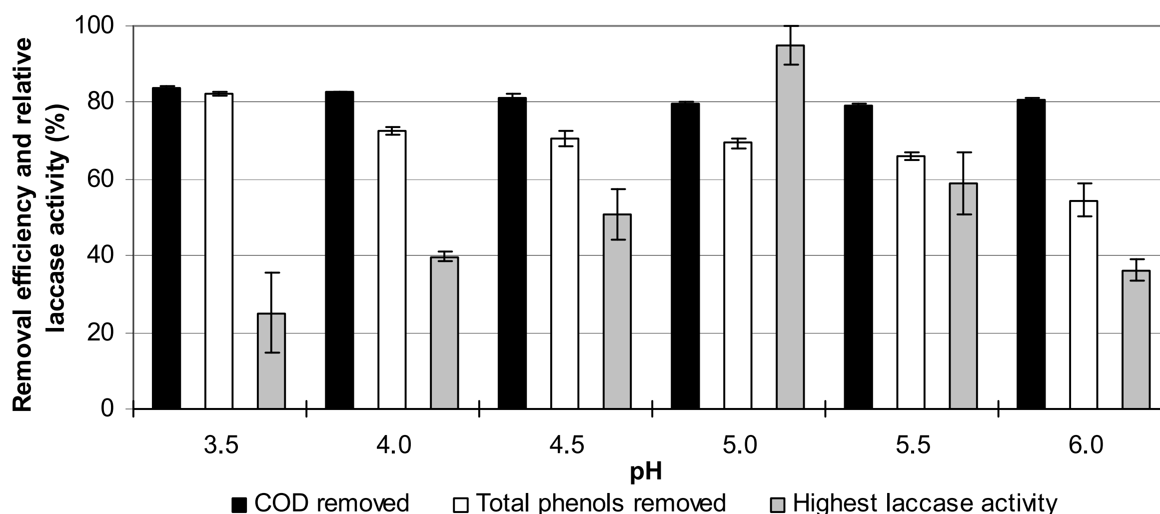


Figure 8.1: Total phenolic compounds and COD removal efficiencies (%) after twelve days and relative percentage of highest laccase activities (normalised against the laccase maximum at pH 5.0 of 6391 ± 332 units/l). Error bars indicate standard deviation.

The removal of COD was consistent for the entire range of pH values, indicating that the same level of degradation of oxidisable matter occurred over the twelve day period in all

flasks. The 80 % removal compares typically to COD removal efficiency for this fungus from this distillery wastewater (79 % removal was previously obtained in Chapter 4).

Total phenolic compounds removal was greatest at the lowest pH value tested and the removal efficiency decreased as the pH became more basic. The increasing pH led to a decrease in total phenolic compounds in the pH 5.5 and 6.0 samples before incubation with *Trametes pubescens*. In the control samples the initial total phenolic compounds concentration decreased by approximately 15 mg/l with increasing the pH from 3.5 to 6.0. Initial values of 285, 285, 286, 286, 284, 276 and 272 mg/l were measured for pH values of 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0 respectively. This was possibly attributable to the increasing negative ions extracting a hydronium ion from the phenolic complexes, resulting in subsequent quinone formation. The colour was visibly greater as the initial pH value was increased, indicating that the small change in total phenolic compounds had led to an increase in colour. Even though there were less phenolic compounds, the phenolic compound removal efficiency of the fungus and laccase activity declined as the pH increased, attributable to less enzyme efficiency above the pH optimum of pH 3.0 to 4.5 (Galhaup *et al*., 2002a).

8.4.2 The effect of different carbon, nitrogen, lignin/cellulose sources and phosphorus

Of the carbon sources tested the best results were obtained with fructose, glucose, sucrose and cellobiose (Table 8.1), which all led to a 1.7-fold increase in laccase concentration relative to the control (no carbon supplement). Lactose and maltose led to a fairly similar increase of 1.5-fold. Only mannitol led to a relative decrease in laccase synthesis, producing just over half of the concentration produced in the control samples. Revankar and Lele (2006b) investigated the effect of different carbon sources on laccase production by *T. versicolor* by assessing glucose, fructose, sucrose, lactose, starch and glycerol as sole carbon sources. They obtained a minimum laccase activity (30 % of the activity using glucose) with fructose, which was contrary to results obtained in this study. Revankar and Lele (2006b) obtained a maximum laccase activity with starch as the sole carbon source (12 % more than glucose). They combined starch and glucose in a 1:1 ratio and obtained a 57 % greater laccase activity (235 U/ml) than had been obtained when using glucose as the sole carbon source. Although they attributed the lower value to glucose repression of laccase synthesis, it could also be that when used in combination with starch, the initial available glucose may be rapidly utilised by the organism for growth. When the glucose is depleted the starch is degraded and utilised slightly less efficiently, but efficient enough to allow for cell maintenance and increased laccase production during the stationary growth phase.

Table 8.1: Results obtained from carbon, nitrogen, lignin/cellulose sources and phosphorus.

		Highest laccase activity (units/l)	Day of highest laccase activity	Relative change (fold)
Carbon sources	Fructose	3160 ± 179	9 ± 0.6	1.7 ± 0.09
	Glucose	3238 ± 793	9 ± 0.6	1.7 ± 0.42
	Mannitol	1091 ± 161	6 ± 0.0	0.6 ± 0.08
	Maltose	2755 ± 188	9 ± 0.0	1.5 ± 0.10
	Sucrose	3239 ± 781	9 ± 0.6	1.7 ± 0.41
	Cellobiose	3306 ± 262	9 ± 0.0	1.7 ± 0.14
	Lactose	2933 ± 164	10 ± 0.6	1.5 ± 0.09
Nitrogen sources	NH_4NO_3	1759 ± 218	6 ± 0.0	0.9 ± 0.11
	NH_4Cl	1521 ± 290	5 ± 0.0	0.8 ± 0.15
	KNO_3	1879 ± 313	10 ± 0.0	1.0 ± 0.16
	H_2NCNH_2	1485 ± 166	5 ± 0.0	0.8 ± 0.09
	Malt extract	2080 ± 489	10 ± 0.0	1.1 ± 0.26
	Yeast extract	2243 ± 35	5 ± 0.0	1.2 ± 0.02
	Peptone	3428 ± 422	5 ± 0.0	1.8 ± 0.22
Lignin / cellulose	Cellulose	1289 ± 220	8 ± 0.6	0.7 ± 0.12
	Bluegum	1919 ± 206	10 ± 0.0	1.0 ± 0.11
	Rooibos	2031 ± 452	9 ± 0.6	1.1 ± 0.24
	Bagasse	1788 ± 288	9 ± 0.0	0.9 ± 0.15
P	H_3PO_4	1978 ± 44	9 ± 1.2	1.0 ± 0.02
	Control	1899 ± 38	10 ± 0.6	1.0 ± 0.02

± Values indicate standard deviation (n = 3)

It was probably the ease of utilisation and incorporation of the carbon source that affected the results obtained for enzyme synthesis. Utilisation was dependent on the chemical structure of the various compounds. Mannitol differs from the other carbon sources in that it was not a ring molecule and was a sugar alcohol (or polyol). Glucose and fructose are simple monosaccharides, while sucrose, lactose, maltose and cellobiose are all disaccharides. Sucrose contains glucose and fructose bonded in a glucose 1α→2 fructose structure. Lactose has a molecular structure consisting of galactose and glucose bonded in a galactose 1β→4 glucose manner. Maltose and cellobiose both contain two glucose molecules. Maltose consists of two α-D-glucose molecules with the alpha bond at C1 of one molecule attached to the oxygen at C4 of the second molecule, which is called a 1α→4 glycosidic linkage. Cellobiose differs from maltose in the configuration of the glycosidic bond and has a 1β→4 linkage as in cellulose. The three disaccharides assessed obtained the same, or slightly lower, laccase increases as the corresponding monosaccharide, indicating that some were easily degraded and assimilated, while the sugar alcohol was poorly utilised.

The nitrogen source that resulted in the greatest laccase synthesis was peptone (1.8-fold increase) (Table 8.1). Of the inorganic nitrogen sources all but the nitrate ions decreased the laccase concentration relative to the controls. This confirmed the results obtained by Galhaup *et al*. (2002b), who observed the greatest laccase synthesis when peptone from meat was added to the growth medium. They found the complex nitrogen source could not be replaced by using asparigine as the sole nitrogen source when using *Trametes pubescens*, as both growth and laccase secretion were negligible. When Fåhraeus and Reinhammar (1967) composed a medium for maximal laccase synthesis using *Trametes versicolor* (shown in Table 8.2), they used asparigine as the primary nitrogen source. Although this appears to highlight the great differences regarding optimal laccase synthesis found between species of the same genus, the primary reason that asparigine was favoured to a complex nitrogen source was that it aided extraction and purification of the enzyme. When Fåhraeus *et al*. (1958) had utilised a complex nitrogen source (yeast extract), the final medium was highly coloured and found to contain larger concentrations of inactive enzyme compared to the corresponding synthetic medium utilising asparigine. The effects of inorganic nitrogen upon laccase synthesis in this study were also corroborated by Revankar and Lele (2006b). They assessed the effect of organic and inorganic nitrogen sources on laccase production using *Trametes versicolor* MTCC 138 and also found inorganic nitrogen sources to yield low laccase activities, while the highest laccase activities were obtained using a complex nitrogen source (yeast extract). Nitrogen supplementation would have to be tested for each species individually as literature exists supporting both high and low nitrogen concentrations for enhancing laccase synthesis. For example, Pointing *et al*. (2000) observed greatest laccase production to occur when using a low nitrogen concentration with a strain of *Pycnoporus sanguineus*, while Galhaup *et al*. (2002b) found a nitrogen-rich medium optimal for laccase synthesis using a strain of *Trametes pubescens*. However, a high nitrogen concentration appears to be generally favoured (Gianfreda *et al*., 1999). Collins and Dobson (1997) demonstrated that increasing the nitrogen concentration in fungal cultures of *Trametes versicolor* resulted in an increase in laccase activity, which corresponded with an increase in laccase gene transcription levels.

Not one of the four cellulose/lignin additions enhanced laccase synthesis. This was not expected, as many plant extracts or lignocellulosic wastes that have been utilised contain tannins and phenolic compounds and have been shown to enhance laccase synthesis (D'Souza *et al*., 2006; Crestini *et al*., 1996). The main constituents of green rooibos (*Aspalathus*

linearis), used to make a tea in South Africa, are phenolic carboxylic and hydroxybenzoic acids. The processed leaves and stems of *A. linearis* also contain hydroxylated benzoic and cinnamic acids, the flavonoids: luteolin, chrysoeriol, quercetin, isoquercetrin, the C-C linked and D-glucopyranosides based on four flavones, but the main constituent is dihydrochalcone aspalathin (van Heerden *et al*., 2003; Rabe *et al*., 1994). Sugarcane bagasse is known to contain an array of phenolic compounds. This includes both ferulic acid and *p*-coumaric acid (Xu *et al*., 2005), which are both known to be potent inducers of laccase synthesis in white-rot fungi (D'Souza *et al*., 2006). The concentration of 1 g/l was possibly too low to allow for an inducer concentration that would have been sufficient to enhance laccase synthesis.

A synthetic solution was designed by combining the results obtained in Table 8.1 with data obtained by Fåhraeus and Reinhammar (1967), who cultured *Trametes versicolor* with the intention of maximising laccase synthesis. This synthetic solution was used to ascertain the effects of a number of inducers that were either added before inoculation or five days thereafter. It was used to test the inducers at two times of addition under optimal conditions as the other phenolic compounds present in the wastewaters may have masked the individual inducer's effect. The components and their concentrations originally used by Fåhraeus and Reinhammar (1967) and those used in subsequent experiments of this study are shown in Table 8.2.

Table 8.2: Components of the synthetic growth medium.

Compound	Fåhraeus and Reinhammar, 1967	Current study
Glucose	20 g	20 g
L - Asparagine	2.5 g	-
D,L Phenylalanine	150 mg	-
Adenine	27.5 mg	-
Peptone	-	3 g
Malt extract	-	3 g
Thiamine HCl	50 µg	-
KH_2PO_4	1 g	1 g
$Na_2HPO_4.2\ H_2O$	100 mg	100 mg
$MgSO_4.7\ H_2O$	500 mg	500 mg
$CaCl_2$	10 mg	10 mg
$FeSO_4.7\ H_2O$	10 mg	10 mg
$MnSO_4.4H_2O$	1 mg	1 mg
$ZnSO_4.7\ H_2O$	1 mg	1 mg
$CuSO_4.5\ H_2O$	2 mg	2 mg

Made up to 1 l with deionised water and pH adjusted to 5.0 using HCl.

8.4.3 The effect of inducers

8.4.3.1 Addition prior to or four days after inoculation

In general the increases in laccase synthesis in this study were greatest when the inducer was added to the medium prior to inoculation. The individual addition of either 2, 5-xylidine, ethanol or copper to the synthetic medium caused the greatest increase of laccase synthesis (Table 8.3).

The greatest inducer of laccase synthesis for both the initial and 96 hour addition resulted from the addition of 2, 5-xylidine, with 3.7 and 2.4-fold increases respectively. Ethanol (2.9-fold increase) and copper (2.4-fold increase) were most beneficial when added prior to inoculation, while 4-methylcatechol and *n*-hydroxypthalamide resulted in greatest increases when added five days after inoculation (both led to a 1.9-fold increase). Gallic acid, tannic acid and quercetin led to a modest improvement in laccase activities when added prior to inoculation (1.4, 1.4 and 1.3-fold increases respectively). Little advantage or negative results were gained by the addition of guaiacol, syringic acid, DBA, phenol, violuric acid, phenol red, cellulose, *p*-coumaric acid, rooibos and *o*-cresol, while negative results were obtained by the addition of DI, HBT and cycloheximide (Table 8.3).

Table 8.3: Results obtained by the addition of inducers at inoculation or five days thereafter.

	Added at inoculation			Added after five days		
Inducer	HLA* (units/l)	Day of HLA	Increase (fold)	HLA (units/l)	Day of HLA	Increase (fold)
2, 5-xylidine	8419	2	3.7	2944	11	2.4
Ethanol	6701	20	2.9	292	6	0.2
Copper	5492	20	2.4	1044	13	0.9
4-methylcatechol	1153	20	0.5	2253	13	1.9
n-hydroxypthalamide	1636	14	0.7	2283	13	1.9
Gallic acid	3303	14	1.4	820	8	0.7
Tannic acid	3114	20	1.4	1111	13	0.9
Quercetin	2966	14	1.3	588	13	0.5
Syringic acid	1862	14	0.8	1404	11	1.2
Guaiacol	2580	16	1.1	1292	11	1.1
DBA	1939	16	0.8	1247	13	1.0
Phenol	2149	14	0.9	1270	11	1.0
Violuric acid	2035	14	0.9	1039	11	0.9
Phenol red	2601	12	1.1	663	13	0.5
Cellulose	2474	16	1.1	328	13	0.3
p-coumaric acid	2370	14	1.0	1227	13	1.0
Rooibos	2119	16	0.9	372	13	0.3
o-cresol	2064	12	0.9	558	11	0.5
DI	127	12	0.1	795	8	0.7
HBT	977	14	0.4	656	13	0.5
Cycloheximide	20	4	0.0	455	5	0.4
Control	2305	12	1.0	1214	13	1.0

*HLA: highest laccase activity

Addition of the inducer prior to inoculation can have greater effects upon the biomass than addition once the biomass is actively growing, as it effectively exposes a lower biomass concentration to potentially toxic compounds for a longer time period. This was evident for 4-methylcatechol where cell growth was visibly retarded and grew in a few large clumps instead of a slurry of fine pellets, as was evident in nearly all other flask cultures. The exposure to cycloheximide was fatally toxic; one day after inoculation the biomass was no more than a fine, milky suspension and no laccase synthesis occurred.

There have been many studies on the effects of inducers using different fungal genera, species and even strains. Differences in laccase induction were already observed in very early studies. Fåhraeus *et al*. (1958) tested a variety of inducers with *Marasmius graminum*, *Marasmius scorodonius*, *Stereum hirsutum* (two strains), *Stereum purpureum*. *Trametes abietinus*, *Trametes hirsutus*, *Trametes zonatus* (two strains) and *Trametes versicolor* (nine strains). Inducers that had previously resulted in good increases with *T. versicolor* improved laccase activities in the other fungi marginally, while only guaiacol induced enzyme synthesis in *S. hirsutum*.

Galhaup *et al*. (2002b) tested seven potential inducers (some of which were the same as this study) at a concentration of 1 mM on an actively growing culture of *T. pubescens* MB 89 in the presence of 2 mM copper. They found gallic acid and catechol to have a marginally positive effect on laccase production, while vanillic acid reduced growth. This study corroborates their gallic acid results. Galhaup and Haltrich (2001) performed a similar study also testing copper and 2, 5-xylidine as inducers. They also found 2, 5-xylidine to stimulate laccase synthesis greatly. However, they found copper to stimulate laccase synthesis to an even greater extent, which contradicts the results found in this study. Strangely, Galhaup *et al*. (2002b) observed no advantage with the addition of 2, 5-xylidine, in direct contradiction to the present study. The reason for this may have been that they were using copper, a very good inducer, at a concentration of 2 mM in their growth medium as opposed to the 0.03 mM used as a background concentration in this study. Although it may be argued that the induction from copper at 2 mM in Galhaup *et al*.'s study resulted in the induction from various aromatic inducers being negligible, further work performed in this chapter (section 8.4.5.5) that combined copper, 2, 5-xylidine and glucose in wastewaters showed the synergistic effect to enhance laccase synthesis tremendously. Copper was tested as an inducer at 1 mM in this study and was shown to be the third best out of the 21 inducers tested.

8.4.3.2 Multiple additions of 2, 5-xylidine

An experiment was conducted to assess what effects the time of addition and of a multiple dosing of 2, 5-xylidine would have upon laccase synthesis of *T. pubescens* MB 89, as it had been shown to elicit the greatest laccase induction of compounds tested. The inducer was tested with multiple additions two days apart as well as variations of the first day of addition. The addition of 2, 5-xylidine at 1 mM was clearly most beneficial when added at the time of inoculation or within a few days thereafter (Table 8.4). The highest relative increases were obtained in samples that had the inducer added before the inoculum or by the fourth day after inoculation. Multiple additions also notably increased the laccase synthesis. Biomass measurements were not taken but it was evident that most of the highest concentrations of laccase were synthesised very early in the experiment, mostly expressed constitutively. This meant that the specific activity would have been greatest for the flasks that obtained extremely high laccase activities early in the experiment, as less biomass had produced very high laccase concentrations. The 2, 5-xylidine had a slight pink/red colour but it was not discernable upon addition to the medium. However, it was rapidly oxidised and the resultant dark, purple product quickly adsorbed to the biomass and turned the solution purple. The reaction resulted in amorphous polymer formation of the 2, 5-xylidine, as most of the purple haze was removed when the samples were centrifuged to remove the biomass. In some of the flasks the purple colouration was completely removed over the course of the experiment, but in general the colour of the solutions was visibly increased due to the addition of 2, 5-xylidine.

Table 8.4: Results obtained with varying 2, 5-xylidine addition.

Days added	Highest laccase activity (units/l)	Day of highest laccase activity	Increase (fold)
0	9999	2	7.7
0+2	11012	3	8.5
0+2+4	13294	7	10.3
2	7529	4	5.8
2+4	7589	4	5.9
2+4+6	7817	4	6.0
4	1972	6	1.5
4+6	3579	9	2.8
4+6+8	5033	13	3.9
6	4058	11	3.1
6+8	3399	9	2.6
6+8+10	3930	9	3.0
8	1454	13	1.1
8	1134	13	0.9
Control	1295	13	1.0

Fåhraeus *et al*. (1958) were the first authors to publish work regarding the inducer effect of 2, 5-xylidine. They tested a variety of fungi and inducers (including guaiacol, indole-3-acteic acid, *o*, *m* and *p*-toluidine, 2, 3-, 2, 4-, 2, 5-, 2, 6-, 3, 4- and 3, 5-xylidine) and found over 160-fold stimulation of laccase synthesis when 2, 5-xylidine was introduced into growing cultures of *T. versicolor*. Fåhraeus *et al*. (1958) observed 2, 5-xylidine to be the most efficient inducer in shake-flask cultures and static-flask culture and the best results were obtained at concentrations of 0.2 mM. The time of addition of was assessed by adding 0.2 mM xylidine at different time intervals in the first six days after inoculation in the shake-flask cultures. In contrast to results obtained in this chapter, they obtained highest activities when the inducer was added three to four days after inoculation. This contrast in results may have been attributable to differences in concentrations and the fungal species that were assessed. Numerous later studies have shown 2, 5-xylidine to be an extremely potent inducer amongst a variety of fungal genera (Rodríguez Couto *et al*., 2002; Pointing *et al*., 2000; Bollag and Leonowicz, 1984), but large variations in effective concentrations have been reported. Tavares *et al*. (2005) found 2, 5-xylidine at a concentration of 0.030 mM in a carbon sufficient media to induce an 8.2-fold increase in *T. versicolor*, while a toxic effect was observed at higher concentrations. Eggert *et al*. (1996b) used 2, 5-xylidine at much higher concentrations (between 10 and 19 mM) and observed a 9-fold enhancement in laccase activity using a strain of *Pycnoporus cinnabarinus*, also observing less stimulation at higher concentrations.

From the results obtained it would appear that to obtain the highest laccase production from *Trametes pubescens* MB 89 the pH at the time of inoculation should be 5.0. A number of carbon sources appear to be equally suitable for enhanced laccase production, but glucose is the standard due to its ready availability and relatively low price. Peptone was the best choice out of both organic and inorganic nitrogen sources. Literature has indicated that different producers and sources of peptone do yield differing results though. Multiple doses of 2, 5-xylidine while the fungus is actively growing led to the greatest stimulation amongst all the inducers and time of addition that were assessed. Copper was not as good an inducer as previously reported for this strain (Galhaup *et al*., 2002b; Galhaup and Haltrich, 2001), but literature has indicated that its induction ability was greatest when used in combination with other inducers when using *Trametes versicolor* (Fåhraeus and Reinhammar, 1967). Combining 2, 5-xylidine with copper would probably enhance the inductive effect and was tested further in the next section in combination with glucose.

8.4.4 Laccase synthesis in supplemented distillery wastewaters and a synthetic wine lees

Raw wastewaters were adjusted to pH 5 and supplemented with either 1 mM copper, 20 g/l glucose, 0.1 % ethanol, three 1 mM 2, 5-xylidine doses 24 hours apart, or treated with a combination of glucose, copper and three 2, 5-xylidine doses. Results obtained are shown in Table 8.5 and Figure 8.2. Table 8.5 contains the averaged activities obtained in all four wastewaters for a particular form of enhancement (pH adjustment, glucose supplementation, copper addition etc.). The highest laccase activities, days upon which the highest laccase activities occurred and relative increases compared to the raw wastewaters are shown. Figure 8.2 displays the averaged highest laccase activity of the triplicate samples for each individual modification tested in each of the four wastewaters separately. Results are discussed further in terms of each adjustment made.

Table 8.5: Averaged data of the results obtained with varying inducer addition to the four wastewaters.

	HLA*	Day of HLA*	Factor increase
Raw	1598	7.8	1.0
pH 5	1947	7.7	1.2
Glucose	1059	11.3	0.7
Ethanol	2284	8.7	1.4
Copper	3055	9.0	1.9
2,5 xylidine	6329	7.2	4.0
2,5 xylidine, glucose and copper	20611	6.1	12.9

*HLA: highest laccase activity

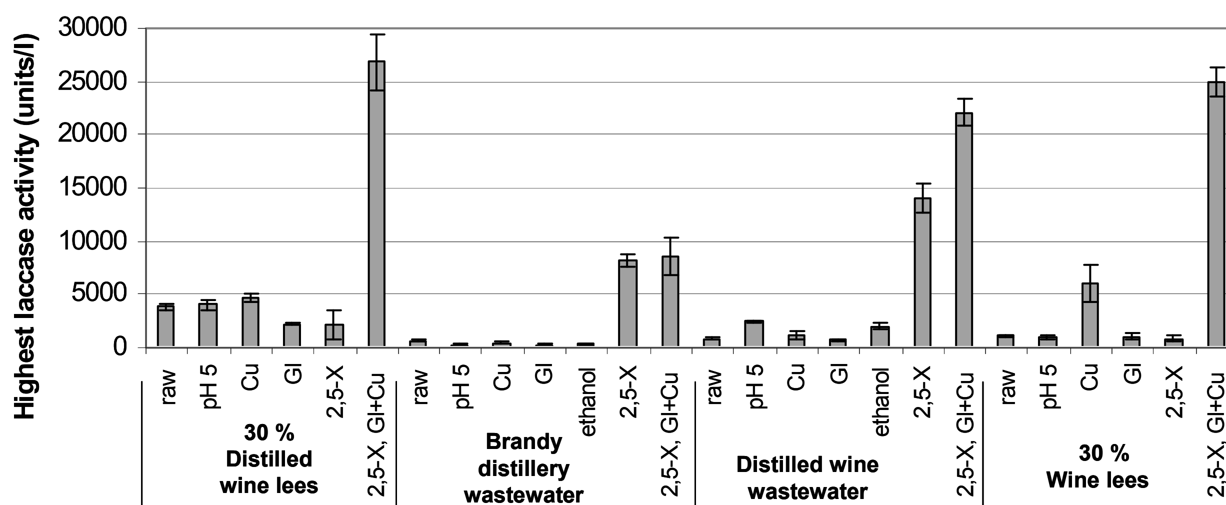


Figure 8.2: Laccase synthesis of *Trametes pubescens* in various supplemented wastewaters. Error bars indicate standard deviation. Raw: unmodified wastewater, pH 5: raw wastewater at pH 5, Cu: additional copper at 1 mM, Gl: additional glucose at 20 g/l, 2, 5-X: additional 2, 5-xylidines added at 1 mM per day for three days and 2, 5-X, Gl+Cu: combination of glucose, copper and 2, 5-xylidine.

The difficulty of comparing and generalising results when comparing the different wastewaters is compounded due to characteristics such as COD and total phenolic compounds being surrogate measurements. The nature of the constituents that exert a wastewater's COD is not indicated by the COD test. The unknowns surrounding these parameters can make a significant difference to wastewater comparison. For example, a wastewater with a high COD comprising mainly easily utilisable sugars rather than more complex, recalcitrant carbon sources would permit higher laccase synthesis than a wastewater consisting primarily of more complex, recalcitrant structures. Similarly, the inductive effect of phenolic compounds varies greatly and induction of laccase would depend on whether the appropriate compounds were present within the total phenolic compounds in a sufficiently high concentration.

8.4.4.1 pH

Laccase synthesis in the raw wastewaters after the pH had been adjusted to 5.0 generally showed little improvement (Table 8.5). The large increase of laccase synthesis that was evident in the pH experiment (Figure 8.1) was not as significant in the wastewaters used in this experiment. The only significant increase was observed in the distilled wine wastewater (Figure 8.2).

8.4.4.2 Copper

Copper addition was of little benefit to any of the distillery wastewaters even though the copper concentrations measured during wastewater characterisation were below 1 mM. Copper is vital to laccase synthesis as the enzyme requires four copper atoms to be catalytically active. The lack of effect may have been attributable to a number of factors that were required together, such as adequate carbon and nitrogen sources that, in conjunction with copper, allow for increased laccase synthesis. Copper addition only proved truly advantageous in the 30 % wine lees (Figure 8.2). Wine lees contains organic acids, sugars and ethanol that can serve as a carbon source and has an extremely high concentration of various phenolic compounds that could induce greater enzyme synthesis. However, even at full strength, the wine lees sample had an extremely low concentration of copper relative to the three distillery wastewaters and this would have been further diminished by the dilution factor to a final concentration of 0.6 µM. Fåhraeus *et al.* (1958) tested the influence of copper concentrations on laccase synthesis by *T. versicolor* and observed very little difference when copper was tested from 0 to 2500 µg/l by itself.

8.4.4.3 Glucose

Glucose addition at 20 g/l displayed no advantage regarding laccase synthesis (Figure 8.2) as all wastewaters that were supplemented with glucose yielded lower laccase activities than the controls. This may have been due to oxygen limitation as the biomass in the glucose-supplemented wastewaters grew in a thick, viscous slurry. When samples were centrifuged to remove biomass it was evident that the growth differed from that in the wastewaters not supplemented with glucose. There was a thick, clear band of mucilaginous growth above the centrifuged pellets, which was consistent with an external polysaccharide layer. When copper and 2, 5-xylidine were included with glucose this thick, transparent layer decreased slightly in size and the solution was visibly less viscous. The combined inducers may have inhibited growth slightly such that it allowed for better oxygen transfer into the shake-flask medium. Moreira *et al*. (1998) have also observed that high glucose concentrations lowered laccase production during secondary metabolism and attributed this to the low pH that resulted from extended primary metabolism.

8.4.4.4 2, 5-xylidine

The addition of 2, 5-xylidine led to the greatest increases in laccase activity in the two distillery wastewaters that were tested at full strength, while leading to no increase in the two wastewaters that were diluted to a 30 % concentration. Although the dilution had been essential to decrease the toxic effects of the wastewater and obtain metabolically active cultures (Chapter 5), the result had been a subsequent dilution of carbon and nitrogen sources and copper. This appeared to negate laccase enhancement, illustrating the synergy of a number of potential factors when trying to obtain high laccase yields with this fungus. There was little notable activity increase in the distilled wine and brandy distillery wastewater for any other inducers tested singly in comparison to the yields obtained with 2, 5-xylidine (Figure 8.2).

Interestingly, the flasks containing 30 % wine lees that were supplemented with 2, 5-xylidine were the only ones in which the fungus grew in a single mass instead of loose pellets and mycelia. Additionally the combination of 2, 5-xylidine in three 1 mM doses spread over three 24-hour periods proved to be the right concentration that resulted in a clear supernatant, while all other flasks containing 2, 5-xylidine had a slight pink to purple colouration by the end of the experiment. The wine lees had the greatest concentration of phenolic compounds

(590 mg/l after dilution), which could have acted as inducers and thereby dampened the effect of 2, 5-xylidine.

8.4.4.5 Copper, glucose and 2, 5-xylidine

Results from this experiment indicated that under the conditions tested, the simultaneous addition of glucose, copper and 2, 5-xylidine resulted in the greatest increase in laccase synthesis. This study also showed that laccase repression that was normally observed in the presence of excess glucose was overridden by the addition of 2, 5-xylidine. Table 8.5 highlights the days on which the highest laccase activities were measured. It was evident that the synergistic effects of enhancing the wastewater with glucose, copper and 2, 5-xylidine also led to very high concentrations of laccase being synthesised early in the experiment. The majority of laccase was synthesised constitutively, instead of occurring during the secondary growth phase. When glucose was added alone the maximum laccase activity occurred much later (11.3 days) than it did when glucose, copper and 2, 5-xylidine were added simultaneously (6.1 days). The phenolic compound caused a shift in laccase synthesis such that the highest values of laccase synthesis were recorded early in the experiment. Synergistic effects using 2, 5-xylidine and other compounds have been observed before. Fåhraeus *et al*. (1958) tested various copper concentrations with and without 2, 5-xylidine and observed very little difference when copper was tested from 0 to 2500 μg/l by itself. However, when tested with xylidine there were very large increases in laccase synthesis when the copper concentration was above 100 μg/l, illustrating the synergistic effect when the two inducers were added simultaneously.

Although there is literature pertaining to the utilisation of wastewaters to produce laccase, there is little demonstrating supplementation of wastewaters in order to enhance the production of laccase by white-rot fungi. However, positive results have been obtained when using a defined growth medium with a wastewater. D'Souza *et al*. (2006) used a marine fungal isolate, NIOCC # 2a, to decolourise wastewaters from paper and pulp mills, textile and dye-making industries and alcohol distilleries. They observed that supplementing a growth medium with lignin and plant derived wastewater residues greatly enhanced laccase synthesis. Black liquor from a paper and pulp mill and molasses spent wash from an alcohol distillery (1 %) enhanced laccase production, while textile wastewater supplementation (1 %) induced laccase synthesis a 100-fold. The marine isolate routinely produced extremely high laccase concentrations (80 units/ml) in batch cultures in a growth medium containing glucose as a carbon source and peptone as a nitrogen source. The values they obtained were much greater

than the values obtained with *T. pubescens* in this experiment, indicating the marine isolate could potentially be better suited to commercial-scale laccase production. It has also previously been shown that agricultural extracts have enhanced laccase synthesis as a variety of phenolic compounds are present (Gómez *et al*., 2005).

8.5 Interim summary and conclusions

A precise model depicting cellular events that control laccase synthesis was not possible. Control of gene expression is one of the most fundamental questions in biology and is a very complex process that is compounded at many levels by multiple factors. Levels of control of gene expression can be exerted in tissue type, cell type, subcellular location of the gene product, timing, environment and molecular elements such as promoter strength and transcription factors. What exasperates a model for laccase synthesis is that not only does phenol-oxidase enzyme occur in all five of the kingdoms, but in the fungal kingdom it has a number of natural functions that include lignification and delignification formation of humic substances in the soil, morphogenesis, and sporulation, growth and development of rhizomorphs, fungal virulence, polymerisation of melanin precursors in differentiated cell walls and detoxification. From this great variety of functions it is no surprise that the cellular mechanisms that govern laccase synthesis are highly complex and interdependent on numerous factors.

In liquid cultures fungal growth varies between loose mycelia and pellets. The growth form affects laccase synthesis and is determined by a number of factors such as strain, inoculum quantity, age and growth rate, growth medium composition, pH, temperature, mechanical shear forces, oxygen availability, polymer additives and surfactants. The presence or absence of an inducer, induction time, nature and composition of the culture medium (constituents that provide a nitrogen and carbon source) and type of culture conditions (oxygen availability, pH of the medium, and temperature of cultivation) also have a strong influence upon laccase production. Regarding *Trametes pubescens* MB 89 prior work had shown that copper and particular carbon and nitrogen sources were essential to higher levels of laccase synthesis. In addition, in prior work it had been demonstrated that laccase was only produced in greater concentrations once the glucose levels had decreased below a critical value. The present study showed that copper concentration was not essential to increasing laccase synthesis (although its presence was essential), and that a particular inducer caused enhanced laccase synthesis

even while in the presence of high glucose concentrations, which countered the previous notion that the majority of laccase is secreted idiophasically and not constitutively.

Conditions tested in this study indicated that a number of factors could greatly increase laccase synthesis using *T. pubescens* in aerobic, agitated cultures. A pH of 5.0 was observed to be the most productive for laccase synthesis. A number of sugars (fructose, glucose, sucrose and cellobiose) were found to stimulate *T. pubescens* equally (1.7-fold increase), while peptone was found to be the most stimulatory of the compounds tested as a nitrogen source (1.7-fold increase). None of the cellulose-containing additives enhanced laccase synthesis. Under the conditions tested, 2, 5-xylidine, ethanol and copper were all observed to be most stimulatory when added prior to inoculation, while 4-methylcatechol and *n*-hydroxypthalamide promoted laccase synthesis significantly when added after the biomass had entered the exponential growth phase. Repeated 2, 5-xylidine dosing early during the experiment led to the greatest increases in laccase activity. Repeated 1 mM doses before inoculation and 24 and 48 hours thereafter in a synthetic medium led to a 12.9-fold increase relative to the control.

The three distillery wastewaters and the wine lees that were tested resulted in highly variable laccase synthesis. The wine lees and the distilled wine lees had both inhibited the growth of *T. pubescens* and had to be substantially diluted to allow for growth. All four wastewaters had greatest laccase increases when copper, 2, 5-xylidine and glucose were all added. The two distillery wastewaters that were tested at full strength had a substantial increase in laccase activity when 2, 5-xylidine was added by itself, while the 30 % wine lees had a substantial increase with the addition of 1 mM copper. Repeated addition of 2, 5-xylidine to the four wastewaters led to an average 4.0-fold increase in laccase synthesis, which was the greatest overall increase with the addition of one inducer. When repeated doses occurred in wastewaters that were supplemented with glucose and copper, a 12.9-fold increase occurred.

The repression of laccase synthesis that is normally associated with the presence of excess glucose was countered by the simultaneous addition of 2, 5-xylidine and copper. Laccase was expressed in greatest concentrations constitutively and decreased shortly after reaching the stationary phase. Generally the greatest laccase expression was observed in the stationary phase and the highest values were recorded near the end of the fermentation period. This has been shown in prior work and literature. This is potentially highly valuable to laccase

production. If a high concentration of the enzyme can be produced very early in the fermentation, the production costs associated with energy and aeration can be halved. Potential contamination, which could ruin a longer fermentation, is less of a concern when the product is produced in half the time of the normal fermentation period.

This work has shown that the large variability between wine-related wastewaters would necessitate investigation into each wastewater individually to assess which supplementation would be most beneficial to laccase synthesis. It appears that no single factor can be altered in order to enhance enzyme synthesis over a broad spectrum of wine-related wastewaters. It was evident that the addition of 2, 5-xylidine was integral to greater laccase synthesis, but this was in addition to other factors present in the wastewaters. The addition of copper, glucose and 2, 5-xylidine had the greatest positive effect on enhanced laccase synthesis, but by the addition of all three components a new medium rather than a supplemented wastewater was created. The viscous slurry of fungal growth would not only hamper mass transfer of nutrients and oxygen, but also decrease the ratio of supernatant to biomass. A low supernatant : biomass ratio would effectively reduce the efficiency of laccase production process as much lower product-containing supernatant would be produced. The most effective strategy would be to adopt a repeated dosage of 2, 5-xylidine with copper, as it would have lower associated costs and the potential for contamination would be less than with the additional inclusion of glucose. Nominal copper concentrations would be added to ensure that the microelement was present as laccase synthesis could not proceed without it. However, using a wastewater for enhanced laccase production would be independent of a wastewater treatment process. The use of both copper and 2, 5-xylidine to enhance laccase synthesis in a wastewater would render it even more of an environmental hazard than the original distillery wastewater.

In conclusion, laccase synthesis in wastewaters by *T. pubescens* can be greatly enhanced by the addition of inducers, pH modification or the supplementation of the carbon source. However, the effectiveness of the inducers varies widely and each wastewater would have to be assessed individually.

Chapter 9

Final discussion and conclusions

9.1 Overall discussion

The hypothesis postulated at the beginning of the study was that the pretreatment of distillery wastewaters using laccase-producing basidiomycetes can lower the COD, colour, total phenolic compounds concentration and increase the pH of wine distillery wastewater, which would render the wastewater more amenable to secondary treatment by anaerobic digestion. Additionally, the phenolic compounds present in the wastewater would induce laccase synthesis, thereby producing high concentrations of a high-value product during wastewater treatment.

The principle of the hypothesis was realised in the initial screening experiment in which the total phenolic compounds concentration, COD and colour were significantly reduced by fungal treatment, while laccase was produced above the target concentration of 1000 units/l by *T. pubescens*. The fungus decreased the COD, the phenolic compounds and the colour, but how the fungal treatment truly managed to aid anaerobic digestion was not in the removal in phenolic compounds, but by raising the pH into the lower end of the range treatable by anaerobic digestion. Anaerobic digestion has a lower limit of pH 6.5. The fungal treatment of the brandy distillery wastewater described in Chapter 4 had raised the pH from below 5.5 to near neutral, a value much more amenable to fungal treatment. The last portion of the hypothesis was also tested by comparing the fungal digestion of raw and PVPP-treated wastewater using *T. pubescens*. Only a small portion of the COD was removed by PVPP treatment, while more than 50 % of the total phenolic compounds were removed. The treatment removing a large percentage of the phenolic compounds greatly diminished the laccase-producing ability of the fungus.

Literature had indicated that phenolic compounds were responsible for inhibition of biological anaerobic treatment systems (Borja *et al*., 1993a; 1993b). Polyvinylpolypyrrolidine pre-treatment of a brandy distillery wastewater did not yield differing data with regards to anaerobic digestion, even though more than half of the phenolic compounds had been removed. The greatest inhibitor appeared to be the rapid drop in pH. There is a diverse array of phenolic compounds in wine-related wastewaters and it could well be that phenolic compounds in wine-related distillery wastewaters and cellar wastewaters may not be as inhibitory as compounds present in other wastewaters such as those generated from sugarcane molasses and olive milling, which are reflected in the literature by the high degree of dilution

required to facilitate biological treatment. Other factors, such as organic acids and inorganic ions also may inhibit anaerobic digestion of distillery wastewaters.

The fungal treatment was an effective pre-treatment system, often attaining COD removal efficiencies greater than 70 %. Similarly phenolic compound and colour removal efficiencies between 60 and 90 % were attained. The quality of the wastewaters after fungal treatment still fell short of the environmental standards required, including a COD of 75 mg/l for environmental release (South African Water Act no. 36, 1998). However, according to the standards of the municipal bylaws of the Nelson Mandela Metropole, the majority of the treated wastewaters were now within the limits for discharge regarding COD (10 g/l), temperature (below 44 ºC) and within the prescribed pH range of 6.0 to 12.0 (Walters, 2007). The only factor that was no longer within municipal discharge parameters was the suspended solids concentration of 1 g/l. This could be improved by either incorporating filtration of the reactor effluent, or by immobilising the biomass. However, the municipal bylaws for the town of Stellenbosch, which is centrally located to most of the wastewater collected, has even stricter stipulations regarding copper, suspended solids and total phenols, although they allow a slightly more acidic wastewater to be discharged (Table 9.1). All of the treated winery wastewaters fell within the parameters for the characteristics that were tested, but most of the distillery wastewaters would require further treatment to allow for unpenalised municipal discharge (Mkize, 2007).

Table 9.1: Acceptable discharge limits according to Stellenbosch municipal bylaws.

Parameter	Acceptable limit
Temperature at point of entry	0 - 40 ºC
Electrical conductivity at 25 ºC	300 mS/m
pH at 25 ºC	5.5 - 11.0
Settleable solids	50 mg/ l
Suspended solids	500 mg/l
Total dissolved solids at 105 ºC	2500 mg/l
Inorganic dissolved solids at 600 ºC	1000 mg/l
*Total phenols as C_6H_5OH	50 mg/l
**Copper as Cu	5 mg/l

*Total phenols include all *o*-, *m*- and *p*-substituted phenols.
**Total collective concentration of all metals may not exceed 20 mg/l.

The data shown in Table 9.2 demonstrate that this study achieved higher removal efficiencies of COD and colour than previous work, and comparable total phenolic compounds removal. These trends, coupled with the generation of laccase, indicate that wastewater treatment with *Trametes pubescens* presents a technically and economically viable method for managing

wine-industry related aqueous wastes. However, the diversity found in alcoholic beverage wastewaters precludes a general conclusion applicable in all cases. Different products generate significantly different wastewaters, which are addressed in the following sections.

Table 9.2: Wastewater treatment and laccase production by *Trametes pubescens* MB 89 in different wastewaters.

Wastewater origin	**Modifications**	**Copper (mg/l)***	**COD (g/l)***	**Total phenols* (mg/l)**	**COD removal efficiency (%)**	**Total phenols removal efficiency (%)**	**Colour removal efficiency (%)**
Brandy 2004	pH 5.3	-	25.5	540	79 ± 1.1	80 ± 4.6	71 ± 1.6
Brandy March 2006	pH 4.5	21.86	29.5	280	77 ± 1.06	79 ± 2.2	86 ± 2.0
Brandy June 2006	pH 4.5	15.54	10.5	35	81 ± 0.03	48 ± 7.8	70 ± 6.5
Amarula	pH 4.5	0.11	26.7	850	75 ± 1.0	88	-50 ± 12
Wine Spirits 1	pH 4.5	5.63	19.9	320	75± 0.05	83 ± 0.3	80 ± 5.5
Wine Spirits 2	pH 4.5 [50 %]	0.17	34.8	290	52± 0.34	76 ± 1.5	74 ± 2.7
Distilled wine lees	pH 4.5 [30 %]	1.69	45.5	540	71± 0.07	60 ± 2.5	77 ± 1.9
Wine lees	pH 4.5 [40 %]	0.12	211.8	1720	79 ± 2.89	87 ± 1.6	82 ± 1.6
WW** samples 1-8	Heat sterilised	-	5.8 -12.5	7 - 95	84 ± 5	62 ± 23	78 ± 18
WW samples 9-16	Heat sterilised	-	0.7 - 4.6	5 - 32	45 ± 26	-47 ± 214	3 ± 50
Literature values							
[1]Whiskey distillery waste	*Geotrichum candidum*			15-58.5	81		
[2]12.5 % molasses spent wash	*Trametes* sp. + supplements						53
[3]Wine distillery and domestic wastewaters	Ozone				23	33	
[4] Shochu distillery wastewater	*Aspergillus terreus* *Geotrichum candidum*		84		42	66 70	
[5]Beet molasses alcoholic fermentation wastewater	*Penicillium decumbens*		82		51-52	70	40
[6]Winery wastewaters	Ozone Followed by activated sludge		24.5		5-25 31-85	17-52	

* Initial readings for full strength wastewaters.
**WW: winery wastewater. Samples 1- to 8 were the 8 highest CODs while 9-16 had the 8 lowest CODs.
[1]Quinn and Marchant (1980)
[2]Fitzgibbon *et al*. (1998)
[3] Beltran *et al*. (2001)
[4]García García *et al*. (1997)
[5]Jiménez *et al*. (2003)
[6] Benitez *et al*. (2003)

9.2 Wastewater treatment

In the screening experiment in Chapter 3 *T. pubescens* showed the most promise from a wastewater treatment and enzyme producing viewpoint. The fungus displayed greater potential for wastewater treatment than *UD4*, *Ceriporiopsis subvermispora* and *Pycnoporus*

cinnabarinus as it achieved the highest COD, total phenolic compounds and colour removal from the raw as well as the PVPP-treated wastewater. *Trametes pubescens* MB 89 also produced the highest concentrations of laccase in the brandy distillery wastewater that was used in the screening experiment. It was for these reasons, rather than economic or operational considerations, that *T. pubescens* was selected for all subsequent work performed in this study.

Treatment of the wastewaters with PVPP prior to fungal inoculation in order to remove potentially inhibitory and colour-containing phenolic compounds had no advantage other than a slight improvement in total phenolic compounds removal, but laccase synthesis in PVPP-treated wastewater was significantly lower (Figure 3.5 page 66). A pre-treatment step using an adsorbent such as PVPP for the removal of phenolic compounds might therefore be useful in the bioremediation of wastewaters containing toxic phenolic compounds, but its use with wine-related distillery wastewaters was not justifiable because the small improvement in the wastewater was offset by the loss of valuable by-product. This contradicts previous literature in which the use of adsorbents has been recommended. For example, activated carbon was used to remove phenol, *o*-cresol, *m*-cresol and *p*-nitrophenol by Ng (1986) in a preliminary treatment that allowed for subsequent aerobic, biological degradation.

Highly varying wastewaters were assessed in this study for biological treatment in order to establish the robustness and applicability of a treatment system that incorporated *Trametes pubescens* MB 89. The fungus proved versatile and achieved a high degree of remediation in most of the wastewaters at full strength. The fungus also synthesised high concentrations of laccase in the distillery wastewaters. All the bioremediation results have been summarised in Table 9.2 and compared to some of the reported values from literature. The remediation results are discussed further for each wastewater type individually.

Bioremediation of the first two brandy distillery wastewaters yielded very good results, while moderate results were obtained for the third wastewater (Chapters 3, 4 and 5). The COD of all three wastewaters decreased by more than 70 %, while the total phenolic compounds decreased by 80 % and the colour was lowered by more than 70 % for the first two wastewaters, which compared well to literature reporting fungal treatment of whiskey distillery wastewater (Quinn and Marchant, 1980). The total phenolic compound removal efficiency for the third wastewater was relatively low (48 %). The reason for the poorer

results obtained with the third wastewater was due to it having a very low initial total phenolics concentration and colour absorbance. Similar results were obtained with the fungal remediation of the winery wastewater samples that had low initial total phenolic compound concentrations and colour (Chapter 7). The brandy distillery wastewater was the most consistent, in that it was always treatable at full strength. It would be the most likely wastewater to be amenable to fungal treatment should a commercial venture be attempted, as it also consistently resulted in the highest laccase synthesis with only a pH modification.

Both wine spirits distillery wastewaters were red in colour and contained moderate total phenolic compound concentrations (>300 mg/l). The first was amenable to fungal treatment and good remediation results were obtained when treated at full strength (Chapter 5). The second wastewater proved more difficult to treat and had to be diluted to a 50 % concentration to prevent fungal inhibition and subsequent remediation. Considering the low concentration of phenolic compounds it seems unlikely that growth inhibition could be attributed to this factor. The slightly higher initial COD was also probably not the cause, as the COD was only 5 g/l above levels which had shown very good results with brandy distillery wastewater tested in the same comparative experiment in Chapter 5. It is likely that an as yet unidentified component of the wastewater was at a concentration that inhibited growth. At 40 % wastewater concentration good remediation and moderate laccase synthesis were achieved. Increasing the pH might have facilitated remediation at greater wastewater concentrations. This idea is supported by Chapter 6, in which increasing the pH by 0.6 units for the Amarula wastewater resulted in good removal efficiencies of both COD and total phenolic compounds. Prior work with the initial brandy distillery wastewater had observed zero biological COD removal at pH 3.9 and 4.5, while the increase to pH 5.3 had resulted in efficient remediation. The increase in pH could have resulted in speciation of metals or changes in organic compounds such that they were no longer at inhibitory concentrations at a higher pH.

The treatment of wine lees by *T. pubescens* MB 89 was severely inhibited at full strength. Wine lees had an extremely high total phenolic compounds concentration of 1780 mg/l, which was double that of the highest distillery wastewater (Amarula wastewater). The majority of these phenolic compounds are derived from extraction from the skin of red grapes and are displayed in Table 2.2 (page 16) in the literature review (Chapter 2). Electrochemistry showed the phenolic compounds in this wastewater to be highly electro-active. Large broad peaks were visible even in dilute samples, which indicated a high concentration of diverse and

highly electro-active species. Although problematic for wastewater treatment systems, these antioxidants are extremely valuable in purified form. The successful extraction of these compounds would result in another valuable by-product and their removal would also aid biological treatment. But as can be seen in Table 2.2, there are many variants of these phenolic antioxidants and successful purification would be a large study in itself.

Although the full strength wine lees was recalcitrant to treatment, the COD removal efficiency at a 40 % wastewater concentration was very good. Even at a 40 % concentration, the COD (85 g/l) was still more than double that of the highest distillery wastewater treated and the colour and total phenolics compound concentration were very high. *Trametes pubescens* still achieved more than 75 % COD removal, 85 % phenolic compounds removal and 80 % colour removal (Chapter 5), which indicated that the compounds present in the wastewater were easily degraded by the complete fungal system when inhibition had been countered by dilution. This showed that wastewaters can be treated even if they do have a high initial COD. One therefore cannot make any assumptions as to the treatability of a wastewater from its COD concentration alone, as the distilled wine lees, which only had a COD of 45 g/l, could only be treated when diluted to 14 g/l. The most obvious potential inhibitor of remediation in the wine lees was the ethanol content. Literature had shown that growth and laccase synthesis of another fungal species (*Pycnoporus cinnabarinus*) were impaired at ethanol concentrations higher than 35 % (Lomascola *et al*., 2003). Additionally the extremely high total phenolic compounds concentration would have contributed to growth inhibition.

Of much greater concern to bioremediation of wine-industry waste in general were the results obtained with the treatment of distilled, extracted wine lees. The distilled wine lees had the highest COD of all distillery wastewaters tested. The wastewater was difficult to treat and had to be diluted to 30 % to enable fungal remediation, which would be a disadvantage for any fungal treatment system. Such a great dilution factor would not be economically feasible as large volumes of potable water would be required. The dilution would also negate the sterility of the distilled wastewater, meaning that the advantage for treatment by a monoculture for the synthesis of laccase would be lost. This suggests that a mixed consortium of organisms would be the most practical option for a biological treatment system as sterility would not be of concern if the only viewpoint was one of bioremediation. A potential method for circumventing the dilution problem would be the mixture of an easily treatable wastewater with the less amenable wastewater. This could be possible where multiple distillations occur

on a single site, which was found to be relatively common in the Western Cape. All three distilleries where samples were collected operated two or more distillation processes at any one time. However, it may be that an inordinately large ratio of amenable : resistant wastewater would be required to obtain a wastewater blend that would be treatable, due to the amenable wastewater itself containing low concentrations of the inhibitory factors, making dilution with it less efficient than it would have been with potable water. This hypothesis could only be tested empirically.

The distilled wine lees contained a much lower phenolics concentration than the wine lees and also contained no ethanol, both of which were factors that could have contributed to fungal inhibition in the original wine lees. This indicated that another factor, possibly ionic strength, was responsible for the inhibitory properties of distilled wine less. Distilled wine lees contained higher COD than the brandy, Amarula and wine spirit distillery wastewaters. It was possible that a portion of the dissolved constituents may have inhibited growth. The phenolic concentration was moderately high for a wine distillery wastewater, but similar to the concentration in the brandy distillery wastewater which had been treatable at full strength and so the concentration of the phenolic compounds alone does not account for the treatability or recalcitrance of the wastewaters. The nature of the compounds is a crucial factor: the phenolic compounds in the distilled wine lees were shown to have a low electro-activity and were resistant to degradation by laccase alone (Table 5.3 page 96), unlike the phenolics in the Amarula wastewater. Electrochemical analysis of phenolic compounds in wastewaters was found to be useful in that it could be related to the potential enzymatic degradation of these compounds by laccase. The work in Chapter 5 showed the wastewaters containing phenolic compounds with lower electro-activities were more susceptible to degradation by laccase. This has not been demonstrated before, and correlates well with the fact that laccase generally has the potential to degrade compounds with an oxidation potential below 0.8 V without the aid of mediators (Camerero *et al*., 2005). Distilled wine lees also served well to reiterate the shortcomings of the enzyme alone versus the whole cell system; whereas less than a third of the phenolic compounds were degraded by laccase, more than two thirds were degraded by the complete fungal system.

The Amarula wastewater contained high COD and the highest concentration of phenolic compounds of all of the distillery wastewaters (Table 9.2). The fungal system treated the wastewater at full strength, lowering the COD by 75 % and the phenolic compounds by 88 %,

both of which were very high removal efficiencies considering the initial concentrations. This is the first reported investigation of the biological treatment of Amarula wastewater. A longer lag phase at the beginning of COD removal occurred at a 100 % Amarula wastewater concentration than had been observed in lower wastewater concentrations or with other wastewaters. Maintenance of a high concentration of biomass in the wastewater would increase the COD removal rate tremendously and eliminate the long lag phase that would occur with a small inoculum. This could be achieved by utilising a continuous flow, steady-state treatment process, which would avoid the slow initial rates of degradation associated with a start-up of a batch reactor. However, effluent clarification may present a problem. When a CSTR was assessed as a potential reactor the fungal culture grew over the baffles entirely within three days after inoculation. This speed of colonisation and subsequent fouling would preclude the use of a membrane for clarification. Gravity settling would not work as the biomass forms pellets that bulk rather than settle. The most cost effective and practical method of solid/liquid separation needs to be identified from dissolved air filtration, filtration, flocculation or immobilisation of the fungus to increase its settleability. What was evident was that centrifugation of pre-treated samples resulted in a completely clear supernatant, which was not achieved when centrifuging the original wastewater as fine pulp remained suspended. This was more than likely due to the growing mycelia adsorbing and breaking down these finer pulp fragments. Kida *et al*. (1995) pretreated shochu distillery wastewater with *Aspergillus awamori* var. *kawachi* and also observed an improvement in filterability of the wastewater after fungal treatment. However, since anaerobic digesters can tolerate loading rates of up to 4.8 kg VSS/m^3/day, other effluent characteristics, such as pH, are more likely to be the limiting factors in subsequent aerobic treatment.

The pH buffering effect of Amarula wastewater concentration was evident when comparing the results obtained with the highest and lowest concentrations treated by *T. pubescens*. By the end of the fungal digestion the pH had been raised by 2.18 ± 0.16 units for the 10 % concentration and only 0.39 ± 0.04 for the 100 % sample. Dilution of the wastewater enabled greater increases of the acidic pH to one more neutral. This would be unlikely to be economically and logistically feasible compared to the addition of a substance such as quicklime ($CaCO_3$) to increase the pH. There is unfortunately no scientific literature regarding the biological treatment of Amarula wastewater. Further investigation into the potential recalcitrance or toxicity of Amarula wastewater to anaerobic digestion would be necessary before one could say that fungal pre-treatment is advantageous. Rather than further treatment,

the effluent may be suitable for agroforestry, as water quality guidelines for non-food crops or processed crops are less stringent than for plants that are directly eaten. In this case screening would be required to remove the suspended solids to prevent clogging of irrigation equipment. According to the municipal discharge limits the wastewater falls within the levels required for copper (<20 mg/l) and COD (<10 g/l), but not within the limits for suspended solids and pH. The receiving soils would have to be tested to establish if the wastewater could be disposed of in this manner.

The most easily treated wastewaters were those from wineries rather than from the distillation process. Autoclaved winery wastewaters were treatable with no modification by a pure culture of *Trametes pubescens* MB 89, achieving up to 91 % COD removal efficiency and 90 % removal of total phenolic compounds (Chapter 6). The results compared well to those obtained by Benitez *et al*. (2003), using ozonation (Table 9.2). The best results regarding COD, phenolic compounds and colour removal efficiencies occurred with the wastewaters that had higher initial values. Unfortunately the fungus secreted or released compounds that were phenolic and added to the colour and the final phenolic compound concentration. One major advantage of fungal treatment was that the pH values increased in all samples from below 6.0 to levels that were within the treatment range of anaerobic digestion after fungal treatment. This meant that the wastewaters could move from a fungal digester to an anaerobic digester with no modification of the pH. However, combining all the wastewaters that are generated in a cellar may have deleterious consequences for a biological treatment system, as rinsing and disinfection water from wine cellars may contain inhibitors such as disinfectants and sanitisers. Ideally wine production wastewaters should be separately delivered to the fungal treatment process via primary screens (to remove the high concentrations of grape stems, seeds, skin and flesh observed in the waste streams) in order to achieve acceptable levels of wastewater treatment.

9.3 Laccase synthesis

9.3.1 Nonsupplemented wastewaters

The distillery wastewaters generated from brandy production (treated in Chapters 3, 4 and 5) were consistently good substrates for high production of laccase. The data for the laccase produced in each wastewater at its highest concentration that was amenable to remediation are shown in Table 9.3. It was shown in Chapter 5 that the two brandy wastewaters contained the highest copper concentrations compared with other distillery wastewaters and the wine lees.

The copper content originates as a result of the distillation occurring in huge copper stills. This would have served to promote laccase synthesis, as copper is a known inducer and a vital component of the laccase enzyme.

Table 9.3: Laccase production by *Trametes pubescens* MB 89 in different wastewaters.

Wastewater origin	Modification	Copper (mg/l)*	COD (g/l)*	Total phenols* (mg/l)	Laccase (units/l)
Brandy 2004	pH 5.3	-	25.5	540	4644
Brandy March 2006	pH 4.5	21.86	29.5	280	8997
Brandy June 2006	pH 4.5	15.54	10.5	35	2847
Amarula	pH 4.5	0.11	26.7	850	1063
Wine Spirits 1	pH 4.5	5.63	19.9	320	3354
Wine Spirits 2	pH 4.5 [50 %]	0.17	34.8	290	1606
Distilled wine lees	pH 4.5 [30 %]	1.69	45.5	540	2043
Wine lees	pH 4.5 [40 %]	0.12	211.8	1720	2929
WW** samples 1-8	Heat sterilised	-	5.8 -12.5	7 - 95	330
WW samples 9-16	Heat sterilised	-	0.7 - 4.6	5 - 32	384
Literature values					
35 g/l ethanol	[1]*Pycnoporus*				266600
2 mM copper	[2]*T. pubescens*				330000
Banana waste	[3]*T. pubescens*				1570
Kiwi fruit waste	[4]*T. hirsuta*				5400
Bagasse powder (2 %)	[5]*T. versicolor*				410
Textile wastewater (1 %)	[6]NIOCC # 2a				86000

[1]Lomascolo *et al*. (2003)
[2]Galhaup *et al*. (2002b)
[3]Osma *et al*. (2007b)
[4]Rosales *et al.* (2005)
[5]Hossain and Anantharaman (2006)
[6]D'Souza *et al*. (2006)
* Initial readings for full strength wastewaters.
**WW: winery wastewater. Samples 1- 8 were the 8 highest CODs while 9-16 had the 8 lowest CODs.

Laccase synthesis was lower when using Amarula wastewater than when *T. pubescens* grew in the brandy and wine spirit wastewaters, but still above 1000 units/l. Laccase activity maxima increased with increasing wastewater concentrations, peaking in the 80 % concentration (1063 ± 26 units/l after 14 days). The 80 % concentration was also the only Amarula concentration in which laccase activities did not reach a maximum and decrease again. The 80 % concentration was probably where the concentrations of compounds needed for laccase synthesis or induction countered the negative effects of growth inhibition that occurred at full strength. The wastewater had an exceptionally high suspended solids content relative to the other wastewaters tested. The majority of the suspended solids were likely to be lignocellulosic in nature, as Amarula wastewater is derived from the juice and pulp of the marula fruit. It would be logical to test other strains in this wastewater, such as *Pycnoporus cinnabarinus* SS3 (Lomascolo *et al*., 2003) or the unidentified isolate (WR-1) (Revankar and

Lele, 2006a). If other strains are shown to have exceptional laccase production under solid state fermentation of lignocellulosic materials they would be ideal candidates to remediate this wastewater and may also produce laccase as a by-product while doing so.

It was hoped that the high concentrations of antioxidant phenolic compounds in the wine lees would induce greater laccase stimulation, while the ethanol would act as a carbon source and laccase inducer. Laccase was synthesised at a high concentration in the wine lees, with activities just below 3000 units/l. The addition of glucose and copper would undoubtedly increase laccase synthesis as both were diluted in order to decrease the growth inhibition effects observed using the full strength wastewater. Although carbon and copper supplementation could be investigated, it is more than likely that the high variability in wine lees would also lead to high variation in the concentrations of laccase obtained. In view of this it is unlikely that laccase synthesis will prove economically reliable considering the dilution, supplementation, sterilisation and downstream processing that would be required to maintain a monoculture and purify the enzyme. It may be more feasible to use sterilised, full-strength wine lees as a supplement to an optimised growth medium instead.

The laccase activity obtained in the distilled wine lees was lower than had been obtained in the wine lees, but higher than obtained in the Amarula wastewater. The large dilution factor required for distilled wine lees would also negate the practicality of utilising this wastewater for enzyme production due to the volumes and voiding of sterility gained by distillation. Strains that have been collected under more extreme conditions, such as the marine-tolerant fungus that D'Souza *et al*. (2006) isolated from decaying wood in mangrove swamps, may display attributes such as halotolerance or possibly increased ionic strength resistance, which would be extremely beneficial for wastewater remediation and possibly laccase synthesis in these wastewaters.

The winery wastewaters were also unsuitable for laccase synthesis. Laccase was synthesised in only five of the sixteen samples and at considerably lower concentrations than in distillery wastewaters (Chapter 7). These low concentrations of laccase were produced even though a few of the samples had relatively high COD values for winery wastewaters, again demonstrating that the measurement of COD alone is insufficient to gauge the potential of a wastewater for laccase synthesis. Some of the COD values were as high as the brandy distillery wastewater in which 2847 units/l laccase was synthesised. Laccase synthesis could

not be related to the parameters assayed for in the characterisation studies, but this does not necessarily mean that there is some as yet undefined factor influencing laccase activity. The enhanced synthesis of laccase is a complex interrelationship between a number of factors. The most likely cause of the lack of laccase synthesis was the lack of appropriate carbon source. Every wastewater collected had been exposed to some microbially-related predigestion, which would have decreased the easily utilisable carbon and nitrogen that was available. Samples were generally collected from pit tanks or runoffs that were exposed to the elements. When the winery wastewaters were examined for the presence of lactose and non-lactose fermenters, there was not one sample that did not contain microorganisms.

9.3.2 Enhancing laccase synthesis

A variety of growth conditions and inducers were tested in order to establish what type of conditions and supplements could be added to wastewater to increase laccase synthesis by utilising data obtained from a synthetic medium. Conditions tested in this study indicated that a number of factors could greatly increase laccase synthesis using *T. pubescens* in aerobic, agitated cultures. The factors that were tested were derived from literature ranging over the past 50 years, with particular attention being paid to the work of Gösta Fåhraeus and Christiane Galhaup. A pH of 5.0 was observed to be the most productive for laccase synthesis. A number of sugars (fructose, glucose, sucrose and cellobiose) were observed to stimulate *T. pubescens* equally, while peptone was found to be the most stimulatory of the compounds tested as a nitrogen source. What was unexpected was that not one of the lignin and cellulose-containing supplements enhanced laccase synthesis. It had been anticipated that due to the difficulty of consumption of these potential carbon sources they would allow for the maintenance of cells into the stationary phase, thereby allowing for greater laccase synthesis. However, this was not observed. It may have been that the excess carbon and nitrogen in the synthetic medium masked any effects of the lignin and cellulosic supplements.

Under the optimal conditions provided in the synthetic medium, ethanol, 2, 5-xylidine and copper were the most stimulatory inducers when added prior to inoculation, while 4-methylcatechol and *n*-hydroxypthalamide promoted laccase synthesis most when added after the biomass had entered the exponential growth phase. The highest relative increases in laccase activity were observed when the inducers were added prior to inoculation and not when added after four days of growth. The two inducers that led to the greatest laccase activity increases when added after four days of growth had been inhibitory (although not

fatally toxic) when added prior to inoculation. It should be stated that the screening of inducers used in this study was a rudimentary technique (although less rudimentary than techniques described in some literature). General methodology in the literature involved testing potential aromatic inducers at one particular concentration between 0.1 and 2 mM and then either adding the inducer prior to inoculation or 4 to 5 days thereafter (De Souza *et al*., 2004; Galhaup and Haltrich, 2001; Farnet *et al*., 1999). Infrequently the effect of the inducer would be assessed at different times of addition (Shuttleworth and Bollag, 1986) and even then it would only be testing a single dose. Ideally a concentration range over three orders of magnitude (such as 0.1, 1 and 10 mM) should be investigated and all should be tested by varying the time additions and number of dosages. The efficacy of inducers has been shown to be highly variable in literature. This is to be expected as the general testing of inducers at a single concentration could be incorrect by at least one order of magnitude with respect to promoting enzyme synthesis or the inducer being consumed as a minor carbon source. Different genera, species and even strains of fungi may react very differently should a range of inducer concentrations be assessed with varying times and numbers of additions. This becomes exceedingly more complex when the synergistic effect of two or more inducers is assessed. The large number of replicates required to ascertain the optimal concentrations and dosages and time of dosage for just one potential inducer causes thorough testing of several inducers and combinations thereof to be an impractical experiment to perform.

The addition of 2, 5-xylidine to the synthetic medium had an interesting effect upon the time of laccase synthesis that opposed literature. Laccase synthesis has been reported to be suppressed when an excess of glucose is available (Galhaup *et al*., 2002b). The addition of 2, 5-xylidine countered glucose repression of laccase synthesis such that high concentrations were secreted constitutively with the multiple dosage and the time-of-dosage variation regime (Chapter 8). The highest laccase activities were measured early in the experiment, indicating that the repression of laccase synthesis that was normally associated with the presence of excess glucose was counteracted by the simultaneous addition of 2, 5-xylidine. Repeated 2, 5-xylidine dosing led to the greatest increases in laccase activity when tested in a optimised synthetic medium. Repeated 1 mM doses before inoculation and continuing as the fungus was actively growing led to a 10.3-fold increase in laccase activity relative to the control. This observation could not be found in the available literature, and led to the experiments to assess the effects of 2, 5 xylidine dosing in wastewaters on laccase synthesis.

A question that arose from literature was whether the inducer was at a concentration that made it act an inducer or as a carbon source. The inducers tested in this study were assayed such that the carbon source concentration grossly exceeded the inducer concentration. Other studies in literature claim that some carbon compounds, such as ethanol, serve as inducers. However, the concentrations at which they are utilised suggest that they were a carbon source. Ethanol would be utilised after the initial growth period and exhaustion of easily utilised carbon sources. Ethanol could maintain the cells such that they can maintain viability and still produce laccase, instead of succumbing to autolysis. This leads to two other factors that were not tested in this study: combinations of carbon sources and the use of continuous culture. Galhaup *et al*. (2002b) had utilised a continuous culture system that maintained the glucose levels of the mature culture at a level just below the concentration that was considered laccase-repressing. By doing so they more than doubled the concentration of laccase produced. It could also be surmised that less easily utilised carbon sources such as starch, ethanol, mannitol etc. may be useful in maintaining cultures once the culture had reached stationary phase and the easily consumed carbon sources (glucose) had been exhausted. This cellular maintenance would possibly increase the length of stationary phase and thereby increase the time allowed for laccase synthesis.

9.3.3 Wastewater supplementation

The three distillery wastewaters and the wine lees that received inducer supplements resulted in highly variable laccase synthesis. The wine lees and the distilled wine lees had both inhibited the growth of *T. pubescens* and had to be diluted to a 40 % concentration allow for growth. All four wastewaters displayed greatest laccase increases when copper, 2, 5-xylidine and glucose were added simultaneously. This was not unexpected as it combined an easily utilisable carbon source, the most potent tested inducer and an inducer that was an integral part of the catalytically-functional enzyme molecule. The 30 % wine lees displayed a substantial increase in laccase activity with the addition of 1 mM copper. The two distillery wastewaters that were tested at full strength had a substantial increase in laccase activity when 2, 5-xylidine was added by itself, which may have been due to the full strength wastewaters not having their potential carbon source decreased by dilution.

The highest laccase synthesis was achieved by multiple dosing of 2, 5-xylidine in wastewaters that were supplemented with glucose and copper, which resulted in a 12.9-fold average increase. The highest laccase synthesis achieved by the addition of a single inducer in the

wastewaters was caused by the repeated dosing of 2, 5-xylidine before, 24 and 48 hours after inoculation and led to an average 4.0-fold increase in laccase synthesis. The repression of laccase synthesis that is normally associated with the presence of excess glucose in these wastewaters was again countered by the addition of 2, 5-xylidine as had been observed using the synthetic medium. Laccase was expressed in greatest concentrations constitutively and decreased shortly after reaching the stationary phase again as it did in the synthetic medium. This was in contrast to results reported in Chapters 3, 4, 5 and 7, where the greatest laccase expression was observed in the stationary phase and the highest values were recorded near the end of the fermentation period. This alone was a very interesting result, which suggests that the cellular mechanism for laccase synthesis can be switched on under normally repressive conditions by the addition of a simple aromatic compound. There is a great potential application for this, as a decreased fermentation period for the generation of a product lowers the costs associated with production. The incorporation of 2, 5-xylidine into the wastewater would be recommended for laccase enhancement, as a final concentration of approximately 0.37 ml/l resulted in large increases in activity.

It would have been interesting to have had the opportunity to assess the effects of 2, 5-xylidine addition in the first brandy distillery wastewater, which was used in Chapters 3 and 4. This wastewater contained a high concentration of soluble compounds and would probably have had a relatively high copper concentration. The effect of 2, 5-xylidine may well have been hindered by the high phenolic compound concentration (the highest observed in all the brandy distillery wastewaters assessed), as had been seen using the diluted wine lees. However, the experiment may have demonstrated that when a sufficiently high carbon source was combined with 2, 5-xylidine (in the presence of copper) high concentrations of constitutively-produced laccase could be induced.

Although effective, the addition of inducers would result in the generation of a new wastewater more toxic than the original. The irony of this project is treating a wastewater to obtain a product and then convert the wastewater into one even more toxic in order to produce higher concentrations of the enzyme and to extract it. Presently the only major application of laccase is in stone-washing of denim. The necessity for the enzyme may be decreased further by genetically adapting microorganisms to produce a polyphenol oxidase, such as by incorporating the laccase gene in a yeast by Larsson *et al*. (2001). There is also the added threat of more efficient microorganisms being discovered that produce higher activities or

more desired isoforms with greater thermostability, ionic tolerance or pH broadness. In addition, the heterologous expression of the laccase genes in other microorganisms may enhance laccase synthesis such that currently utilised and developed techniques may be made instantly obsolete.

One question which remains unanswered is “what is the role of laccase?”. The answer to this question lies not so much in the enzyme’s ability to oxidise phenolic compounds, but in the insufficiencies in the classification of laccase. “Laccase” as we currently define it has a number of roles. Various laccase enzymes from fungal sources have a broad substrate range, making it difficult to classify/sub-classify the enzyme. The classification of laccase as a polyphenol oxidase incorporates too large and diverse a group of enzymes that are capable of oxidising phenolic compounds. Laccase oxidises mono and diphenols, but does not oxidise tyrosine (the defining difference between laccase and tyrosinase). There is one compound, syringaldazine or N,N-bis (3,5-dimethoxy-4-hydroxybenzylidene hydrazine), which is often considered to be a unique substrate of the laccase enzyme (Harkin *et al*., 1974). However, even the oxidation of this substrate overlaps with that of the peroxidases, hence requiring the presence of catalase or EDTA when assessing enzyme activity in unpurified broths. A table in a highly consequential review by Petr Baldrian (2005) shows the amazing variation in laccase purified from different fungal sources. Molecular masses of the purified enzyme range from 43 to 383 kDa, optimum temperatures range from 25 to 80 C, pIs from 2.6 to 6.9, optimum pH values from 2.0 to 5.0, the K_M for syringaldazine ranges from 3 to 4307 µM, while the K_{cat} values for syringaldazine range from 16800 to 26800 s^{-1}. The K_M and K_M for 2, 6 dimethoxyphenol range from 26 to 14720 µM and from 100 to 360000 s^{-1} respectively. The level of glycosylation was also shown to be highly variable, ranging from 1 to 80 % of the mass (Baldrian, 2006). With ranges like these in the fungal laccases alone it appears evident that the enzyme is in need in subclassification.

9.4 Conclusions

1) **Screen selected fungal strains to obtain a species that grows and produces laccase in distillery or winery wastewater.**

Trametes pubescens showed the most promise from a wastewater treatment and enzyme producing viewpoint. The fungus displayed better potential for wastewater treatment than *UD4*, *Ceriporiopsis subvermispora* and *Pycnoporus cinnabarinus*, and was shown to grow and produce laccase in a brandy distillery wastewater.

2) Reduce the total phenolic compounds concentration by the catalytic function of laccase and fungal degradation in the distillery and winery wastewaters.

High colour removal efficiencies were attained by the submerged *T. pubescens* culture, while the greatest removal by laccase was just above 10 %. The most dramatic results were obtained in the wine lees dilutions and this serves as the best example to illustrate the differences between fungal and enzymatic treatment. The wine lees contained the highest levels of phenolic and electroactive compounds. Although laccase treatment resulted in reasonable total phenolics decreases, the colour was significantly increased, which indicated that the new compounds formed by laccase were more colour-rich than the parent compounds. Interesting results were obtained when anaerobically digesting winery wastewaters that had been fungally pre-treated. Phenolic compounds that had not been degraded by the fungal treatment were removed by the anaerobic treatment.

3) Compare the action of an enzymatic versus fungal treatment system.

Four different wastewaters were inoculated with *T. pubescens* or spiked with laccase in order to compare fungal and enzymatic degradation of phenolic compounds and colour removal. The complete fungal system was observed to be far superior to enzymatic treatment alone. Enzymatic treatment reduced the total phenolic concentrations but did little to improve the colour of the wastewaters, and in the case of more phenolic-rich wastewaters laccase treatment significantly increased the colour. The maximum degradation of phenolic compounds by the fungal culture was generally much higher than the maximum degradation by the enzyme alone.

4) Reduce the COD of the distillery and winery wastewaters to levels tolerable to anaerobic microorganisms and degrade compounds that may inhibit anaerobic digestion.

An inference of the hypothesis was that fungal treatment would render the wastewater more easily treatable by a secondary biological treatment step, such as anaerobic digestion, due to the removal of potentially toxic phenolic compounds. It was not conclusively shown that the removal of the phenolic compounds rendered the wastewater more amenable to secondary treatment by anaerobic digestion. Although literature had indicated that the phenolic compounds were inhibitory to biological treatment systems, this was not found in this study using a brandy distillery wastewater. This was most evident in the two control samples of the experiment comparing the anaerobic digestion of distillery wastewaters that were raw, PVPP and fungally-pre-treated. The controls compared a raw wastewater to a wastewater from

which more than half of the phenolic compounds had been removed by PVPP. The metabolic activity displayed by a rapid initial change in pH and COD removal were very similar for the two control samples. There was no indication that the phenolic compounds inhibited metabolism. This was later corroborated in Chapter 6 when work comparing anaerobic digestion of raw and fungally-pre-treated winery wastewaters showed no advantage regarding final COD removal efficiencies arising from the pre-treatment step. This part of the hypothesis can therefore be rejected.

5) Reduce the colour of the distillery and winery wastewaters.

Colour removal efficiency of 70 % or greater was attained in all the distillery wastewaters other than Amarula wastewater and the eight winery wastewaters with lower initial COD concentrations. The decrease in colour correlated well to the removal of phenolic compounds.

6) Produce laccase at a relatively high concentration of >1000 units/l.

Laccase was produced in concentrations greater than 12000 units/l in the brandy distillery wastewater at full strength, with no modifications other than a pH increase using sodium carbonate.

7) Demonstrate that the remediation and laccase production can be scaled up using an airlift, bubble-lift or stirred tank reactor.

A bioreactor capable of treating 50 l of wastewater per batch was constructed and assessed. Although laccase was produced in high concentrations, the wastewater treatment performance was less effective than had been achieved in the flask studies due to a flaw with the air filtration system. Due to moisture collecting in the air filter there were two periods of inefficient oxygen transfer and mixing, which detrimentally affected the aerobic treatment system. The experiment could unfortunately not be repeated because the reactor was on loan for a limited period of time, and the volume of wastewater donated was sufficient for one trial. The scale-up results are very promising but cannot be considered conclusive.

8) Demonstrate that laccase production can be enhanced by wastewater supplementation.

High concentrations of laccase were achieved using some of the distillery wastewaters. It was found that more factors than solely the presence of phenolic compounds were responsible for high laccase synthesis. A combination of a particular carbon source and inducers was required for enhanced laccase synthesis. The hypothesis that the pre-treatment of distillery wastewaters

using laccase-producing white-rot fungi would lower the COD, colour, total phenol concentration and increase the pH can be accepted based on this study. Laccase synthesis by *T. pubescens* growing in wastewater can be greatly enhanced by the addition of inducers, pH modification or the supplementation of the carbon source. Using the fungus to remediate wastewaters originating from distilled wine and Amarula production is possible. However, the effectiveness of the inducers varied widely and each wastewater type would have to be assessed individually. The high variation in wastewater characteristics from batch to batch (as observed in Table 5.1) would complicate the long term utilisation of these wastewaters for commercial laccase production, even utilising wastewater from a single source.

9.5 Recommendations for further work

Through the course of this study it became evident that an intensive treatment system requiring a monoculture of *Trametes pubescens* MB 89 would not be a viable method for wastewater treatment. The technical knowledge and stringent sterility requirements to maintain a monoculture would not be financially justifiable. Further work in this area would require a more robust system that could maintain bioremediation in the presence of various other microorganisms such as yeasts and bacteria. The key to a successful wastewater pre-treatment would probably lie with a system that utilises immobilised biomass treating a well oxygenated or thin layer of wastewater. The incorporation of white-rot fungi mycelia within a RBC, such as the one developed by Malandra *et al*. (2004), may well enable such a technology to withstand perturbations caused by higher phenolic compound-containing loads. Enzymes secreted by the fungi could possibly result in less inhibition of other microorganisms if phenolic compounds truly are the cause of inhibition. Immobilisation studies or the investigation of an RBC would explore the application of *T. pubescens* in wastewater treatment further.

It was more than evident that some of the wastewaters were toxic to the fungus when tested at full strength and the toxicity could not be ascribed to phenolic compounds. A parameter such as 'total phenols' is highly misrepresentative, as some phenolic compounds are non-reactive while others are acutely toxic. A problem that has not yet been investigated is the effect of ionic concentration upon the white-rot fungi's ability to remain metabolically active in distillery wastewaters. Additionally, the effects of increased ionic strength upon a variety of microorganisms such as anaerobic bacteria need to be established. This may assist in providing the toxic range of a characteristic such as electrical conductivity that may be

directly related to the inhibition potential of the wastewater. However, the problem of conductivity, COD etc. being surrogate measurements negates their usefulness as a variety of factors (including the soluble organic matter in the form of fruit acids) affect the ionic strength of the medium. Additionally, different organic acids with differing dissociation constants would have different effects in wastewaters and are dependent on both temperature and pH. Wastewaters should be assessed according to a biological toxicity index. Several tools to assess the toxicity of wastewaters to biological treatment have been developed and are commercially available (e.g. Amtox, Microtox, Cellsense, Toxalert and others) and can be used in conjunction with measurements of chemical component and quantitative structure activity and property activity relationships (QSAR/QPAR) modelling to develop more accurate predictions of wine industry wastewaters' behaviour under different treatment conditions. Indeed such combined measurement approaches are promoted under the Whole Effluent Toxicity (WET) and Direct Toxicity Assessment (DTA) legislation and guidelines in the USA and the UK. Whole effluent toxicity data would be of greater realistic value as they would be far more representative of the effect that the wastewater would have upon environmental microorganisms should the wastewater be discharged onto soil or into a water body. Additionally, once a toxicity weighting has been established for a surrogate or chemical measurement, the individual components making up a wastewater may be established to allow for more accurate toxicity or inhibitory predictions.

The concentrations and characteristics of organic acids in these wastewaters also warrant further investigation. It is one thing to know their ratios and their concentrations, but it is not known whether they have a role in inhibiting biological treatment systems. Does the fungal treatment increase the pH and lower the buffering effect enough to decrease the requirement for alkaline compounds to a level which represents a financial benefit to a secondary biological treatment system such as anaerobic digestion? It is not known whether the buffering effect that the organic acids provides allows for better pH stabilisation after the pH has been adjusted by other means, or whether the degradation of these acids is responsible for shifts in pH.

There are a few major factors that would affect the financial viability of this project with respect to *T. pubescens* and these wine-related wastewaters being used for laccase production. These are: product demand, costs associated with media purchase and preparation, organism maintenance and sterility, capital equipment, process energy, downstream processing,

packaging and marketing. Although a distillery wastewater may be utilised to reduce the costs associated with media purchase and sterilisation, its use has shortcomings. The major factor is the variability in laccase production. A specimen will have to be obtained that even under the worst production, will maintain sufficiently high laccase synthesis to produce a profitable laccase concentration. Once a particular wastewater has been selected (in this study brandy distillery wastewater appeared to be the most likely candidate), closer scrutiny of the wastewater characteristics that may lead to these inconsistencies will enable supplementation that minimises variation in production. After the major initial cost of capital equipment, the two largest operational costs will be associated with energy to maintain the process and with downstream product processing. Energy consumption will have to be kept to a minimum, but is not something that can be altered to a great extent once minimised. The large amount of compounds present in the wastewater after fermentation would necessitate a larger investment in downstream product processing should the enzyme be required with a high degree of purity. Downstream processing, however, presents many opportunities for cost-cutting. Although a crudely purified laccase is obtainable with a double precipitation of ammonium sulphate (Fåhraeus and Reinhammar, 1967), more environmentally sound methods need to be investigated, notably foam fractionation. Utilisation of less hazardous purification techniques such as foam fractionation should be investigated in these wastewaters as this technique has the potential to extract the enzyme using only 1 to 2 mM of a detergent cetyltrimethylammonium bromide (CTAB) (Linke *et al*., 2007) instead of 750 g/l of ammonium sulphate. Additionally the utilisation of an airlift reactor provides the perfect basis for batch extraction using foam fractionation. It may require only the addition of CTAB and a change in aeration from large aeration bubbles to fine flotation bubbles to recover the enzyme.

The idea behind this project was novel, but the target enzyme production limit that would make it economically feasible has not been identified. A study of that sort is highly complex as it would require a sound knowledge of industrial engineering and economic studies for global use and demand, factoring in global increases in demand should the enzyme become available more cheaply. As far as the production of laccase is concerned the technology developed in this study shows that the hypothesis was validated and that laccase could be produced at over 12000 units/l, requiring only a modification in pH if using a brandy distillery wastewater.

Chapter 10

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Appendix A.1
Increase in removal of phenolic compounds in a wine distillery wastewater with the addition of phenol.

A red wine (Tassenburg) was diluted to 20 % concentration. The diluted wine (100 ml) was added to 500 ml flasks, autoclaved and then aseptically inoculated with *T. pubescens* MB 89. Pure phenol was added to two of the flasks at 750 mg/l and two flasks with 5 mM distilled water served as a control. Flasks were shaken at 150 rpm at 28 °C for 10 days and were tested in duplicate. Almost all phenolic compounds were removed when 750 mg/l phenol was added to the solution (Table A.1)

Table A.1: Total phenolic compounds concentration in water (mg/l), in a 20 % wine concentration and in a 20 % wine concentration with 750 mg/l pure phenol.

Day	Water a	Water b	20 % (a)	20 % (b)	20 % + 750 mg/l phenol (a)	20 % + 750 mg/l phenol (b)
0	2	2	272	272	959	959
1	3	4	95	95	215	323
2	0	1	78	81	18	18
3	3	5	72	79	22	28
5	8	11	55	52	15	21
6	5	7	43	49	13	11
8	6	10	42	53	19	17
10	8	12	30	35	11	11
Removal efficiency			89 %	87 %	99 %	99 %

Appendix A.2

Determination of the concentration of phenolic compounds that could be desorbed from various resins using 1M HCL, 1M NaOH, ethyl acetate, ethanol and heat.

Various anion exchange resins were weighed in Eppendorf containers (50 mg for Cellex D and 100 mg for all other resins, to within 1 mg) and then exposed to 1 ml of distilled winery wastewater. Samples were vortexed every three minutes over a fifteen minute period, centrifuged and the supernatant aspirated and analysed for total phenolic compounds to determine the concentration in solution.

Resins were rinsed with deionised water and then exposed to 1ml of the potential desorbant for five minutes. Hydrochloric acid (1 M), sodium hydroxide (1 M), ethyl acetated and absolute ethanol were all assessed as potential desorbants. Heat treatment was assessed by placing the resins into 1 ml of deionised water and autoclaving for 5 minutes (100 ºC at 121 kPa). All samples were centrifuged and supernatants were assayed for phenol content.

The results clearly indicate that a large portion of the phenolic compounds are not desorbed from the resin, indicating that they were strongly bonded.

Table A.2: Results of phenols adsorbed and desorbed (ng/100 mg resin).

Resin	Total adsorbed	Desorbed in HCl	Desorbed in NaOH	Desorbed in autoclave	Desorbed in ethyl acetate	Desorbed in ethanol
De- Acidite H	175	40	69	39	59	45
AG 1-X2	581	48	53	38	35	95
AG 1-X8	224	57	61	40	5	44
AG 3-X4	166	77	79	36	20	74
Cellex D	291	171	185	83	42	94
PVPP	526	64	268	66	43	133

Original concentration of phenolic compounds in wastewater was 730 mg/l.

Appendix B

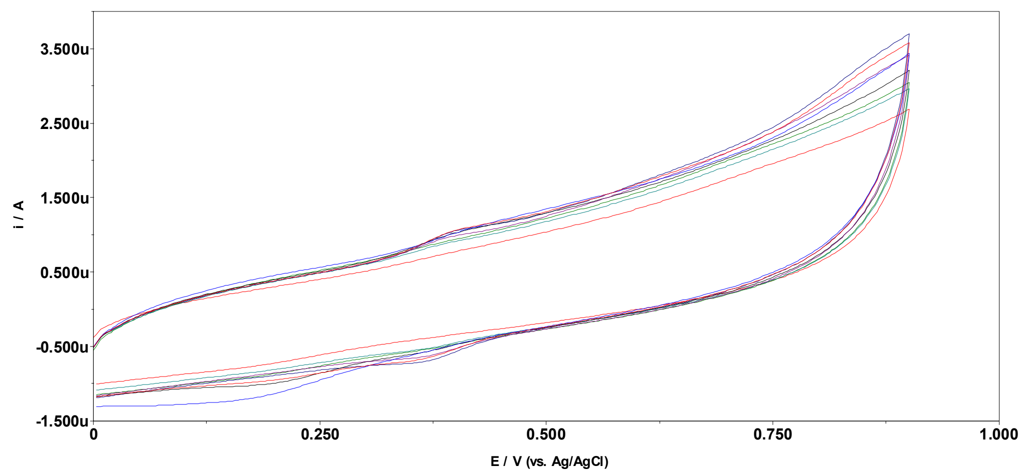

Figure B.1: Results from CV (CV vs. Ag/AgCl) of wastewater B1 showing a small peak at 0.4V.

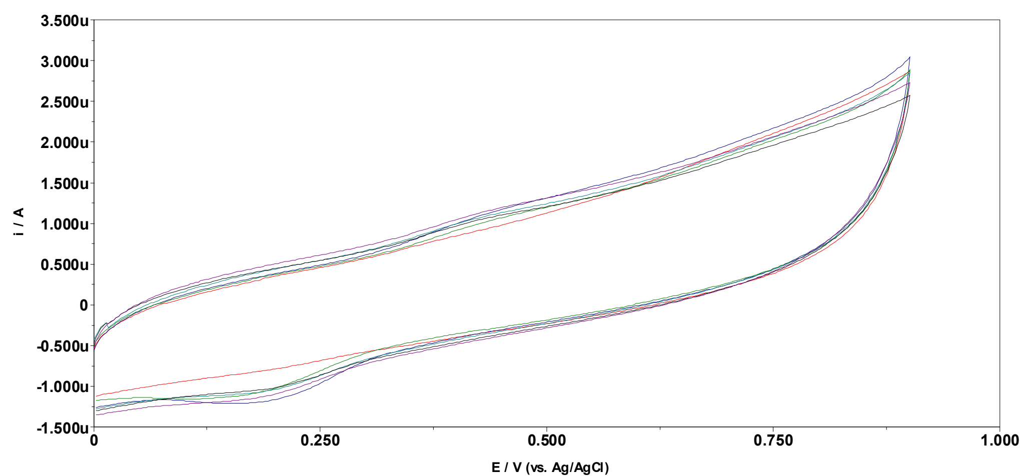

Figure B.2: Results from CV (CV vs. Ag/AgCl) of wastewater B2 showing a small peak at 0.4V.

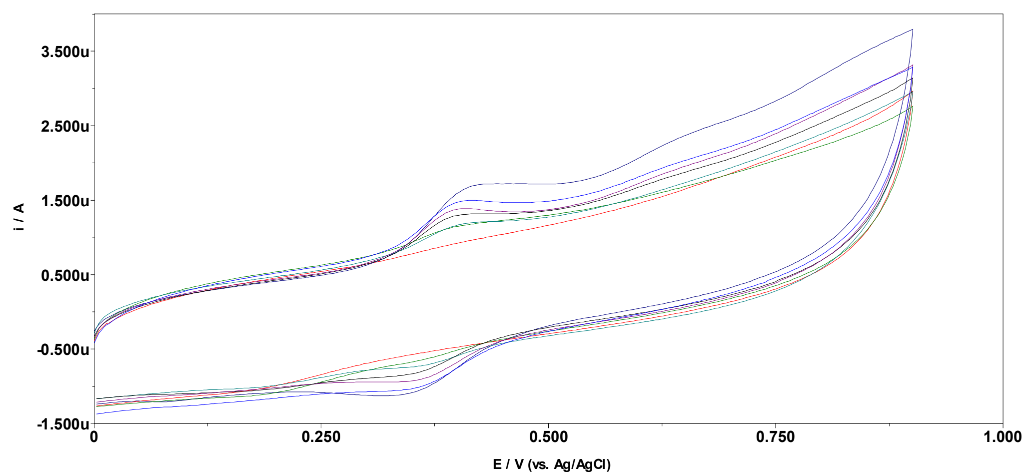

Figure B.3: Results from CV (CV vs. Ag/AgCl) of wastewater S1 showing two peaks, indicating two electro-active compound present in this sample.

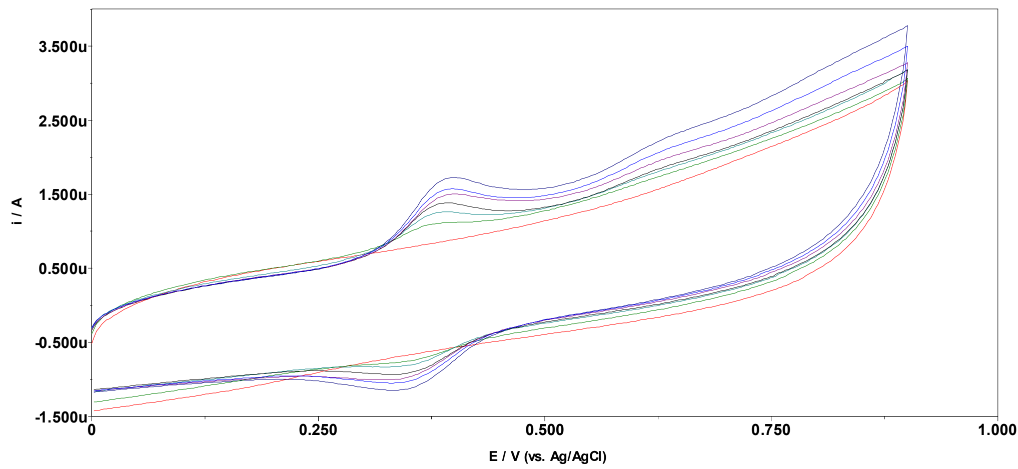

Figure B.4: Results from CV (CV vs. Ag/AgCl) of wastewater S2 showing a small peak at 0.395V.

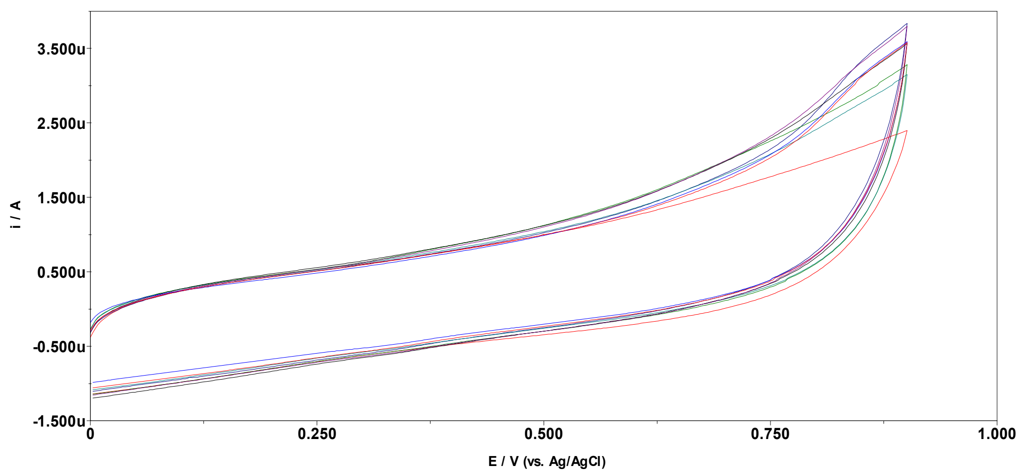

Figure B.5: Results from CV (CV vs. Ag/AgCl) of wastewater DL. The lack of any distinguishable peaks indicates no electro-active compounds were present.

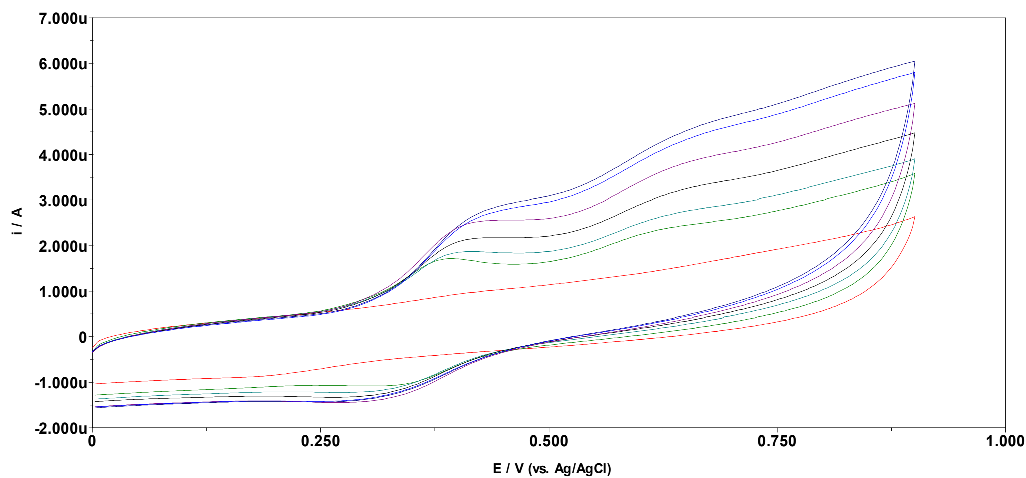

Figure B.6: Results from CV (CV vs. Ag/AgCl) of wastewater L showing a major peak at about 0.4V and high concentrations of electro-active compounds at higher potentials.

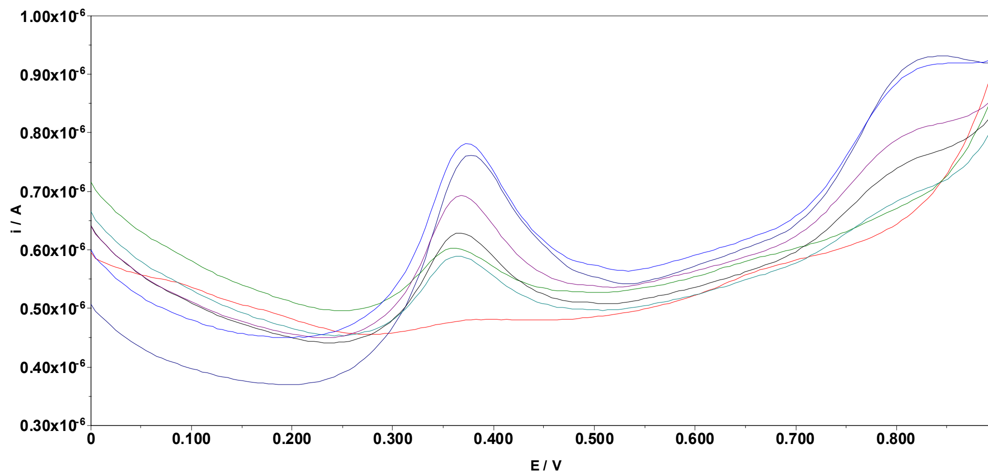

Figure B.7: Results from DPPV (DPPV vs. Ag/AgCl) of wastewater B1 showing small peaks at 0.38V and at 0.8V indicating low concentrations of electro-active compounds.

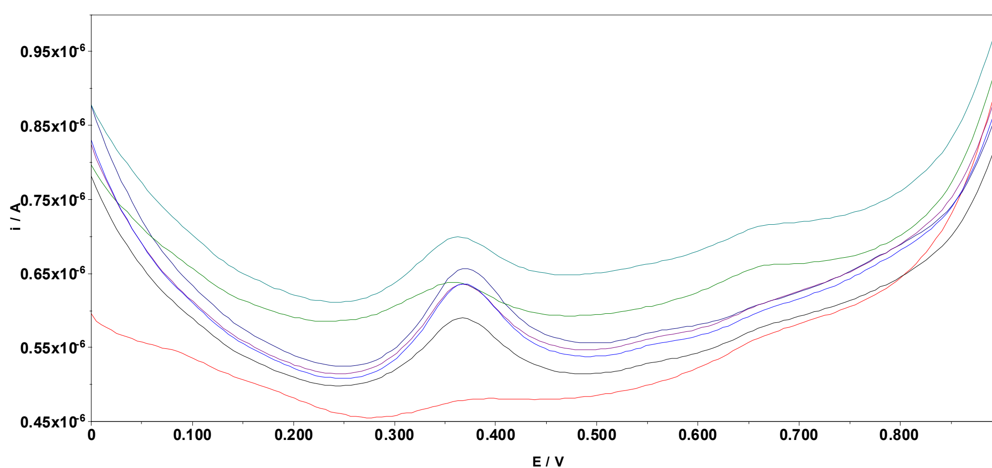

Figure B.8: Results from DPPV (DPPV vs. Ag/AgCl) of wastewater B2 showing peaks at 0.38V and one at 0.68V, but at very low concentrations.

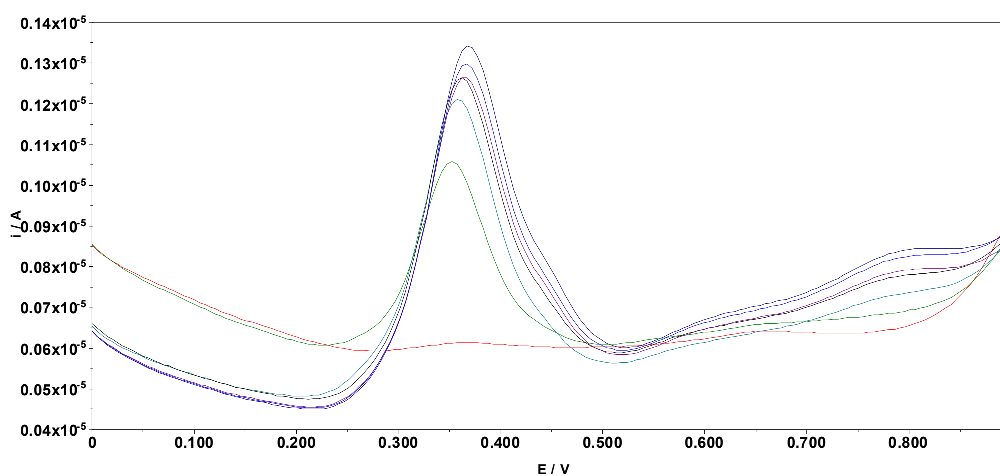

Figure B.9: Results from DPPV (DPPV vs. Ag/AgCl) of wastewater S1 showing a highly electro-active compound at 0.37V. Another peak at 0.8V was only detectable at higher concentrations.

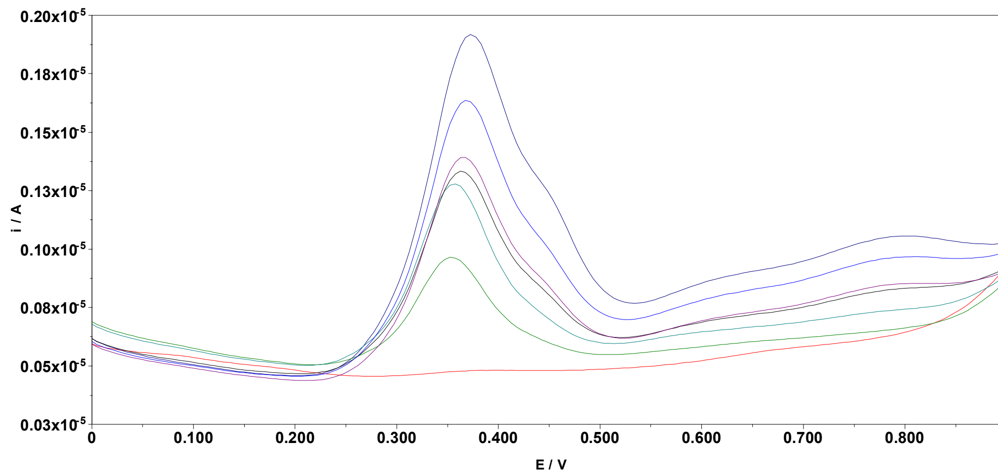

Figure B.10: Results from DPPV (DPPV vs. Ag/AgCl) of wastewater S2 showing a small peak at 0.38V. More than one electro-active compound was present (due to the width and the bump to the right of peak).

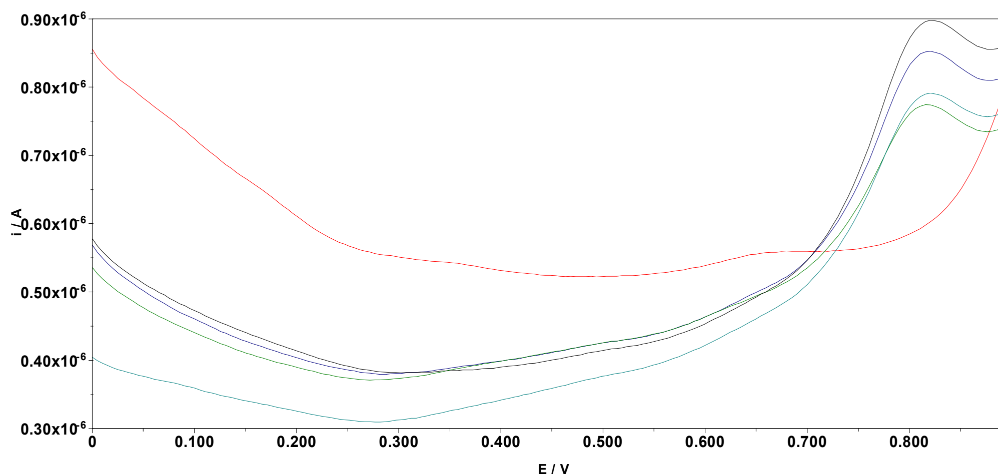

Figure B.11: Results from DPPV (DPPV vs. Ag/AgCl) of wastewater DL showing an electro-active compound 0.8V, but it is only evident at higher concentrations.

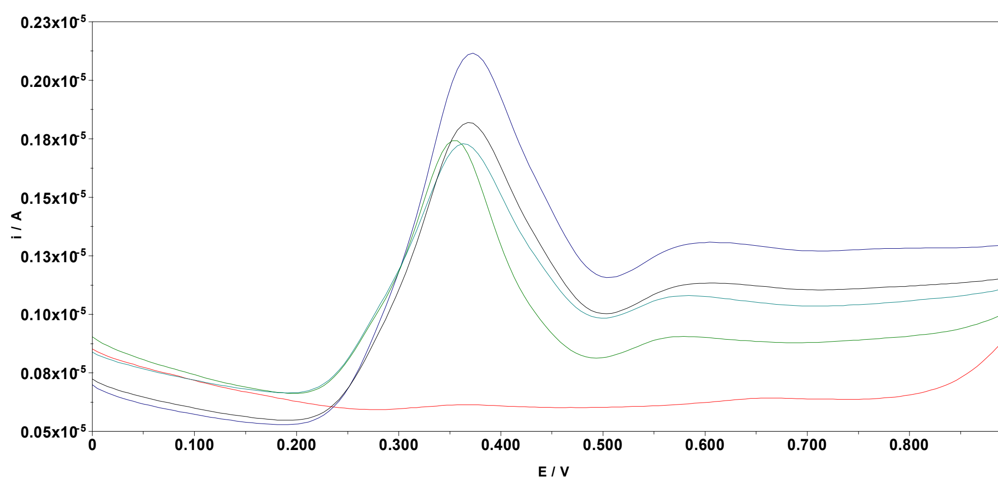

Figure B.12: Results from DPPV (DPPV vs. Ag/AgCl) of wastewater L showing a very broad peak at 0.38V. This sample contained highly electro-active compounds.

Appendix C

(Reproduced from website http://www.marula.org.za/techfruit.htm without alteration)

Technical Info: fruit pulp

Fruit Pulp | Oil | Literature Review

MNP produces a highly refined fruit pulp as a base for the food and beverage industries instead of alcoholic and soft drinks. With a unique and pleasurable taste, an attractive colour and odour and health properties such as high vitamin C and potassium, this new fruit product has all the physical requirements of the growing fruit based drinks industry. Marula, *Scelerocarya birrea*, subspecies caffera, is one of Africa's botanical treasures. Steeped in legends, it has a rich and diverse cultural history, plus natural heritage as an indigenous, wild organic African berry.

More background information on Marula Fruit Pulp.

Marula Pulp Characteristics
Of the wide range of nutrients in the Marula pulp the Vitamin C content has attracted the most attention. Indeed the vitamin C content is important to local communities who know well that it prevents scurvy. It has on average 3 - 4 times the Vitamin C of Orange with up to 194mg per 100g.

The fruit has small amounts of other vitamins such as thiamine, riboflavin and nicotinic acid. It is 85% moisture and 14% carbohydrate, mostly sucrose. Citric acid is the most abundant acid excluding ascorbic acid but malic and tartaric acid have also been noted by scientists. The mineral composition of the fruit shows high concentrations of Potassium, Calcium and Magnesium. A group of panellists best described the aroma of Marula to be like that of grapefruit and there are compounds that the two fruits have in common. However, the similarity in taste between grapefruit and marula is probably because of a dominant bitter taste caused by non volatiles. The smell of marula juice has also been liken to pineapple but this is probably also only in part due to complimentary volatile components such as ethyl acetate, benzaldehyde, linalool.

Marula Natural Product Pty Ltd's Product Characteristic

The total soluble solids of Marula fruit pulp varies between 7.5¡Brix and 15.5¡Brix. Our average value for the 2003 fruit season was 12.1¡Brix. Climate has a large influence on the soluble solids content of Marula and Marula Natural Products concentrates its fruit pulp activities towards then end of the fruit season to bring you the best quality juice.

The vitamin C content of our 2002 Marula Pulp sample was 112.3mg/100ml and a frozen sample from the 2001 season (12 months old at the time) showed a vitamin C content of 106.1mg/100ml and so we are confident that most of the vitamin C content is preserved during freezing, storage and shipping.