

THE MICROBIAL PRODUCTION OF POLYPHENOL OXIDASE
ENZYME SYSTEMS AND THEIR APPLICATION IN
THE TREATMENT OF PHENOLIC WASTEWATERS

THESIS

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TABLE OF CONTENTS

	Page
DECLARATION	iv
ABSTRACT	v
ACKNOWLEDGEMENTS	vi
DEDICATION	vii
LIST OF TABLES	viii
LIST OF FIGURES	x
LIST OF PLATES	xv
 CHAPTER 1: INTRODUCTION: PHENOLIC EFFLUENTS - TREATMENT AND DISPOSAL	 1
1.1. DEPHENOLIZATION PROCESSES	3
1.2. ENZYME-CATALYZED BIOTRANSFORMATIONS IN ORGANIC SOLVENTS	33
1.3. THE ASSIMILATION OF PHENOLIC COMPOUNDS BY MICROORGANISMS	38
1.4. RESEARCH OBJECTIVES	46
 CHAPTER 2: MATERIALS AND METHODS	 48
2.1. The selection of microorganisms	48
2.2. Growth on solid substrate (plate tests)	48
2.3. Enzyme production in batch culture systems	49
2.4. Enzyme induction studies	50
2.5. Enzyme production over time	55
2.6. Deep liquid culture of <i>Agaricus bisporus</i> mycelia	55
2.7. Mushroom compost as a source of tyrosinase	56
2.8. Enzyme extraction from batch culture systems	56
2.9. Crude tyrosinase extraction from <i>Agaricus bisporus</i>	57
2.10. Spot tests	58
2.11. Enzyme assays	58
2.12. The determination of protein concentration	60
2.13. Zymograms as a detection system for tyrosinase	60
2.14. Preliminary enzyme purification using salt fractionation	61
2.15. Characteristics of <i>Agaricus bisporus</i> crude tyrosinase	62
2.16. Enzyme inhibition studies	62
2.17. Kinetics of <i>Agaricus bisporus</i> crude tyrosinase	63
2.18. Phenol removal efficiencies	63

2.19.	Enzyme catalysis in organic media	65
2.20.	Product analysis	66
CHAPTER 3: SCREENING MICROORGANISMS FOR TYROSINASE		73
3.1.	INTRODUCTION	73
3.2.	RESULTS	75
3.3.	DISCUSSION	92
3.4.	CONCLUSIONS	104
CHAPTER 4: <i>STREPTOMYCES</i> AS A SOURCE OF TYROSINASE		106
4.1.	INTRODUCTION	106
4.2.	RESULTS	113
4.3.	DISCUSSION	147
4.4.	CONCLUSIONS	158
CHAPTER 5: <i>Agaricus bisporus</i> TYROSINASE		161
5.1.	INTRODUCTION	161
5.2.	RESULTS	164
5.3.	DISCUSSION	195
5.4.	CONCLUSIONS	216
CHAPTER 6: THE USE OF WHOLE CELL SYSTEMS FOR THE TREATMENT OF PHENOLIC EFFLUENTS		218
6.1.	INTRODUCTION	218
6.2.	MATERIALS AND METHODS	221
6.3.	RESULTS	224
6.4.	DISCUSSION	234
6.5.	CONCLUSIONS	236
CHAPTER 7: GENERAL DISCUSSION		237
REFERENCES		248
APPENDIX 1 - MICROORGANISMS SELECTED FOR SCREENING AND SELECTIVE MEDIA		A-1
APPENDIX 2 - MEDIA FOR THE CULTURING OF SAMPLES		A-3

APPENDIX 3 - GLUCOSE-PHENOL MINIMAL MEDIA	A-5
APPENDIX 4 - MEDIA FOR CULTURING <i>Streptomyces</i>	A-6
APPENDIX 5 - THE SOMOGYI-NELSON METHOD FOR GLUCOSE DETERMINATION	A-9
APPENDIX 6 - PROTEIN DETERMINATIONS	A-11
APPENDIX 7 - ENZYME ACTIVITY GELS (ZYMOGRAMS)	A-13
APPENDIX 8 - SALT FRACTIONATION	A-15
APPENDIX 9	A-17

DECLARATION

In accordance with the regulations for the award of the degree of Doctor of Philosophy, I declare that the work presented in this thesis is my own original research. Assistance with experimental procedure has been declared elsewhere when necessary. Neither the whole, nor any part of the thesis, has been, is being, or will be submitted for a higher degree from any other university.

Research projects initiated from this study are listed in appendix 9.

ABSTRACT

Phenolic compounds are a group of organic chemicals present in the wastewaters of many synthetic industrial processes. Due to their extreme toxicity to man and animals, and deleterious impact on the environment, a range of techniques exist for the effective treatment and disposal of these pollutants. Biological degradation using microbial enzymes presents a valuable alternative to conventional wastewater treatment systems.

This research was therefore initiated to investigate the polyphenol oxidase enzyme system and the feasibility of its application for effluent treatment and studies in organic solvents.

The enzyme system is widely distributed in nature, with *Agaricus bisporus* (the common mushroom) being the best known producer. Biochemical investigations of the enzyme system were therefore carried out using this extract. A screening programme was initiated to identify microbial polyphenol oxidase producers which could be cultured in liquid media, thereby enabling the production of large quantities of enzyme in fermentation systems. Extensive growth optimization and enzyme induction and optimization studies were carried out on selected cultures.

A number of good producers were isolated, namely a bacterial culture designated AECI culture no. 26, *Streptomyces antibioticus*, *Streptomyces glaucescens* and a manipulated strain, *Streptomyces lividans* (pIJ702). Enzyme production by *Agaricus bisporus* mycelia was optimized in deep-liquid culture; enzyme extracts showed high phenol removal efficiencies.

Streptomyces antibioticus, *Streptomyces glaucescens*, *Streptomyces lividans* (pIJ702) and AECI culture no. 26 whole cells were also investigated for phenol-removing ability in simulated phenolic effluents. The use of whole cells reduces enzyme inactivation and instability due to the protection of the enzyme system within the cell. All cultures showed improved removal efficiencies in phenolic growth media. These results strongly suggest their use for phenol removal in continuous systems.

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DEDICATION

I would like to dedicate this thesis to my parents, who
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LIST OF TABLES

	Page
Table 1.1: A cost analysis of three dephenolization systems (1980 US dollar) . .	7
Table 1.2: Laccase and tyrosinase substrate specificities (adapted from Käärik, 1965; Sjöblad and Bollag, 1981).	13
Table 1.3: Characteristics of laccase, tyrosinase and peroxidase enzymes (Sjöblad and Bollag, 1981).	13
Table 3.1: Growth of bacterial and fungal cultures on glucose-phenol agar plates.	76
Table 3.2: Growth of environmental isolates on glucose-phenol agar plates. . . .	77
Table 3.3: Enzyme activity studies (activity expressed as units.ml ⁻¹ extract). . . .	79
Table 3.4: Enzyme induction by ethylene (enzyme activity expressed as units.ml ⁻¹ extract).	80
Table 3.5: Enzyme induction by the inclusion of metabolic inhibitors (extracts assayed against tyrosine and activities expressed as units.ml ⁻¹ extract).	81
Table 3.6: Protein determinations of extracted samples.	83
Table 3.7: An examination of enzyme inhibition (results expressed as units of activity.ml ⁻¹ extract).	86
Table 4.1: Results of <i>Streptomyces</i> shaker-flask cultures B1 and B2 tyrosinase assayed against phenol and tyrosine (units of activity.ml ⁻¹).	114
Table 4.2: Results of <i>Streptomyces</i> shaker-flask cultures S1 tyrosinase assayed against phenol and tyrosine (units of activity.ml ⁻¹).	114
Table 4.3: Extracellular tyrosinase production of <i>Streptomyces</i> cultures grown in pH 7.2 GYM media expressed as units of enzyme activity.ml ⁻¹ . .	123
Table 4.4: Protein determinations of extracted samples.	125
Table 4.5: <i>S. antibioticus</i> and <i>S. glaucescens</i> tyrosinase production in pH 7.2 GYM media calculated as units of enzyme activity.ml ⁻¹	127
Table 4.6: Biomass production by <i>S. lividans</i> (pIJ702) in pH 7.2 GYM medium.	129

Table 4.7:	Biomass production of <i>S. lividans</i> (pIJ702) cultures at different pHs.	135
Table 4.8:	<i>S. lividans</i> (pIJ702) tyrosinase activities assayed against L-DOPA (units of activity.ml ⁻¹) after ammonium sulphate purification.	137
Table 4.9:	Tyrosinase activities (units of activity.ml ⁻¹) in pH 7.6 MMT cultures assayed against L-DOPA.	146
Table 4.10:	R _f values for bands of a variety of <i>S. lividans</i> (pIJ702) tyrosinase preparations resolved on zymograms.	147
Table 4.11:	A comparison of published and experimental results.	151
Table 5.1:	The stability of crude tyrosinase (units.ml ⁻¹ extract).	166
Table 5.2:	The influence of temperature on enzyme activity (units.ml ⁻¹ extract).	167
Table 5.3:	A substrate specificity profile for crude mushroom tyrosinase (units.ml ⁻¹ extract).	167
Table 5.4:	Kinetic constants for crude tyrosinase on three substrates.	171
Table 5.5:	Results (units of activity.ml ⁻¹) of mushroom mycelial culture extracts assayed against L-tyrosine.	194
Table 6.1:	Cultures selected for whole cell fermentation studies and their respective activities against L-tyrosine and L-DOPA, expressed as units of enzyme activity.ml ⁻¹	221
Table 6.2:	Culture media and conditions of growth for optimum enzyme production by cultures selected for whole cell fermentations.	222
Table 6.3:	Increase in biomass production after 7 days, expressed as a percentage.	228
Table 6.4:	Phenol removal efficiencies (expressed as % phenol removed) after 7 days as determined by GC analysis.	234

LIST OF FIGURES

	Page
Figure 1.1: The formation of aryloxy radicals from phenol (Sjoblad and Bollag, 1981).	10
Figure 1.2: Tyrosine oxidation by <i>N. crassa</i> tyrosinase (Horowitz <i>et al.</i> , 1970).	22
Figure 1.3: Scale drawings depicting configurations of the cresolase-active copper-oxygen complex. The large circles represent copper atoms, the small circles oxygen (Matthew and Parpia, 1971).	27
Figure 1.4: The pathway of tyrosinase oxidation in the browning reaction (Matthew and Parpia, 1971).	30
Figure 1.5: The reaction of polyphenol oxidases in organic solvents (Kazandjian and Klibanov, 1985).	37
Figure 1.6: Benzene derivatives degraded via catechol (Cripps and Watkinson, 1977).	40
Figure 1.7: The ortho- pathway for catechol and protocatechuate degradation in <i>P. putida</i> (Orriston and Stanier, 1966).	41
Figure 1.8: Oxidation of monohydric phenols to pyruvate by the meta- cleavage pathway (Ribbons, 1970).	42
Figure 3.1: A positive L-tyrosine enzyme assay result.	82
Figure 3.2: A profile of tyrosinase production by <i>N. vasinfecta</i>	85
Figure 3.3: Phenol removal efficiency of Hogsback culture no. 1 (extracellular extract, tyrosinase induced by oxytetracycline) as monitored by GC from 0 to 5 hours.	87
Figure 3.4: Phenol removal efficiency of Hogsback culture no. 1 (extracellular extract) induced by oxytetracycline.	88
Figure 3.5: Phenol removal efficiency of AECI culture no. 32 (extracellular extract) as monitored by GC from 0 to 5 hours.	89
Figure 3.6: Phenol removal efficiency of AECI culture no. 32 (extracellular extract).	90
Figure 3.7: Phenol removal efficiency of AECI culture no. 26 (intracellular extract) as monitored by GC from 0 to 5 hours.	91

Figure 3.8:	Phenol removal efficiency of AECI culture no. 26 (intracellular extract).	92
Figure 4.1:	Circular linkage map of <i>S. glaucescens</i> (Baumann and Kocher, 1974).	110
Figure 4.2:	Restriction map of pIJ702 (Katz <i>et al.</i> , 1983).	112
Figure 4.3:	Phenol removal efficiency of <i>A. bisporus</i> crude tyrosinase (dried extract) at 0 and 4 hours.	116
Figure 4.4:	Phenol removal efficiency of <i>S. antibioticus</i> (B2 intracellular aqueous extract) at 0 and 5 hours.	117
Figure 4.5:	Phenol removal efficiency of <i>S. antibioticus</i> (B2 intracellular dried extract) at 0 and 6 hours.	118
Figure 4.6:	A comparison of phenol removal efficiencies of <i>S. antibioticus</i> extracts and <i>A. bisporus</i> crude tyrosinase.	119
Figure 4.7:	Phenol removal efficiency of <i>S. lividans</i> (pIJ702) (B2 intracellular dried extract) at 0 and 6 hours.	120
Figure 4.8:	A comparison of phenol removal efficiencies of <i>S. antibioticus</i> , <i>S. lividans</i> (pIJ702) and <i>A. bisporus</i> crude tyrosinase.	121
Figure 4.9:	A comparison of biomass production by <i>S. antibioticus</i> at pH 7.2 and pH 9.	122
Figure 4.10:	A comparison of biomass production by <i>S. antibioticus</i> and <i>S. glaucescens</i> in pH 7.2 GYM media.	122
Figure 4.11:	<i>S. antibioticus</i> in pH 7.2 GYM medium.	124
Figure 4.12:	<i>S. glaucescens</i> in pH 7.2 GYM medium.	124
Figure 4.13:	The relationship between enzyme activity and biomass production for <i>S. glaucescens</i> in pH 7.2 GYM medium.	127
Figure 4.14:	The relationship between enzyme activity and biomass production for <i>S. antibioticus</i> in pH 7.2 GYM medium.	128
Figure 4.15:	Biomass production by <i>S. lividans</i> (pIJ702) in pH 7.2 GYM medium.	130
Figure 4.16:	Tyrosinase production by <i>S. lividans</i> (pIJ702) in pH 8 MMT medium.	132
Figure 4.17a:	Extracellular extract of <i>S. lividans</i> (pIJ702) in pH 8 MMT medium (homogeneous sample).	134

Figure 4.17b:	Extracellular extract of <i>S. lividans</i> (pIJ702) in pH 8 MMT medium (extract from clear zone of settled sample).	135
Figure 4.18:	Enzyme activities of <i>S. lividans</i> (pIJ702) extracts grown in MMT medium at various pH values and assayed against L-DOPA.	136
Figure 4.19:	Enzyme activities of <i>S. lividans</i> (pIJ702) pH 7.6 extracts assayed against L-DOPA after salt purification.	138
Figure 4.20:	Extracellular extract of <i>S. lividans</i> (pIJ702) in pH 7.2 MMT medium (homogeneous sample).	142
Figure 4.21:	<i>S. antibioticus</i> in pH 7.6 MMT medium.	143
Figure 4.22:	<i>S. glaucescens</i> in pH 7.6 MMT medium.	144
Figure 4.23:	<i>S. lividans</i> (pIJ702) in pH 7.6 MMT medium.	144
Figure 4.24:	Biomass production in pH 7.6 MMT media.	145
Figure 5.1:	Crude tyrosinase assayed against monophenolic substrates.	165
Figure 5.2:	Crude tyrosinase assayed against L-DOPA.	165
Figure 5.3:	Michaelis-Menten (A) and Lineweaver-Burke (B) plots for crude tyrosinase assayed against L-tyrosine.	168
Figure 5.4:	Michaelis-Menten (A) and Lineweaver-Burke (B) plots for crude tyrosinase assayed against L-DOPA.	169
Figure 5.5:	Michaelis-Menten (A) and Lineweaver-Burke (B) plots for crude tyrosinase assayed against p-cresol.	170
Figure 5.6:	A comparison of changes in phenol concentration as determined by GC analysis for samples analyzed immediately after sampling and after storage (161 000 units crude tyrosinase added to reaction medium).	175
Figure 5.7:	The UV analysis of samples analyzed immediately upon completion of a phenol removal experiment (161 000 units crude tyrosinase added to reaction medium).	176
Figure 5.8:	The UV analysis of samples analyzed after storage (161 000 units crude tyrosinase added to reaction medium).	176
Figure 5.9:	A comparison of phenol removal efficiency of 14 500 units crude tyrosinase at two different pH values (GC analysis).	177

Figure 5.10:	The UV analysis of samples analyzed immediately upon completion of a phenol removal experiment carried out at pH 8 (14 500 units crude tyrosinase added to reaction medium).	178
Figure 5.11:	The UV analysis of samples analyzed immediately upon completion of a phenol removal experiment carried out at pH 6 (14 500 units crude tyrosinase added to reaction medium).	178
Figure 5.12:	Enzyme-quinone binding - GC determination of quinone concentration after 0, 2 and 4 hours.	180
Figure 5.13:	Pilot-scale salt fractionation experiment of crude tyrosinase.	182
Figure 5.14:	Changes in phenol and catechol concentrations as shown by the GC analysis of samples taken during enzyme catalysis an aerated organic medium (14 500 units of crude tyrosinase added to reaction medium).	184
Figure 5.15:	The UV analysis of samples taken during an enzyme catalysis in an aerated organic medium (14 500 units of crude tyrosinase added to reaction medium).	184
Figure 5.16:	A catechol spiking experiment to confirm the presence of catechol.	185
Figure 5.17:	Changes in phenol concentration as shown by the GC analysis of samples taken during enzyme catalysis in an unaerated organic medium (14 500 units of crude tyrosinase added to reaction medium).	186
Figure 5.18:	The UV analysis of samples taken during enzyme catalysis in an unaerated organic medium (14 500 units of crude tyrosinase added to reaction medium).	187
Figure 5.19:	Changes in phenol concentration (indicated by area under peak) as shown by the GC analysis of samples taken during a modified organic aeration experiment using 2 755 units of crude lyophilized tyrosinase.	187
Figure 5.20:	A comparison of phenol removal ability of crude and commercial tyrosinase (GC analysis).	191
Figure 5.21:	The UV analysis of samples taken during an aeration experiment using 14 500 units commercial tyrosinase.	191
Figure 5.22:	An example of fluctuating product patterns detected on assay.	205
Figure 5.23:	A proposed binding-release mechanism for tyrosinase in aqueous media.	206

Figure 6.1:	Biomass production of AECI culture no. 26 in phenol-containing growth medium.	224
Figure 6.2:	Biomass production of AECI culture no. 26 in phenolic buffer. . . .	224
Figure 6.3:	Biomass production of <i>S. lividans</i> (pIJ702) in phenol-containing growth medium.	225
Figure 6.4:	Biomass production of <i>S. lividans</i> (pIJ702) in phenolic buffer.	225
Figure 6.5:	Biomass production of <i>S. glaucescens</i> in phenol-containing growth medium.	226
Figure 6.6:	Biomass production of <i>S. glaucescens</i> in phenolic buffer.	226
Figure 6.7:	Biomass production of <i>S. antibioticus</i> in phenol-containing growth medium.	227
Figure 6.8:	Biomass production of <i>S. antibioticus</i> in phenolic buffer.	227
Figure 6.9a:	Changes in phenol concentration of AECI culture no. 26 0.1% phenol-containing growth medium as monitored by GC.	229
Figure 6.9b:	Changes in phenol concentration of AECI culture no. 26 1% phenol-containing growth medium as monitored by GC.	229
Figure 6.10a:	Changes in phenol concentration of <i>S. lividans</i> (pIJ602) 0.1% phenol-containing growth medium as monitored by GC.	230
Figure 6.10b:	Changes in phenol concentration of <i>S. lividans</i> (pIJ702) 1% phenol-containing growth medium as monitored by GC.	230
Figure 6.11a:	Changes in phenol concentration of <i>S. glaucescens</i> 0.1% phenol-containing growth medium as monitored by GC.	231
Figure 6.11b:	Changes in phenol concentration of <i>S. glaucescens</i> 1% phenol-containing growth medium as monitored by GC.	231
Figure 6.12a:	Changes in phenol concentration of <i>S. antibioticus</i> 0.1% phenol-containing growth medium as monitored by GC.	232
Figure 6.12b:	Changes in phenol concentration of <i>S. antibioticus</i> 1% phenol-containing growth medium as monitored by GC.	232
Figure 6.13a:	Changes in phenol concentration of <i>S. antibioticus</i> and <i>S. glaucescens</i> 0.1% phenolic buffer.	233
Figure 6.13b:	Changes in phenol concentration of <i>S. antibioticus</i> and <i>S. glaucescens</i> 1% phenolic buffer.	233

LIST OF PLATES

	Page
Plate 2.1: Apparatus used for aeration experiments.	64
Plate 3.1: Growth of <i>S. glaucescens</i> on tyrosine-containing agar.	78
Plate 3.2: Growth of <i>S. antibioticus</i> on tyrosine-containing agar.	78
Plate 3.3: Growth of <i>S. lividans</i> (pIJ702) on tyrosine-containing agar.	78
Plate 3.4: Zymogram developed against L-tyrosine.	84
Plate 4.1: Zymogram developed against L-DOPA.	126
Plate 4.2: A change of MMT medium colour from clear to dark blue on inoculation with <i>S. lividans</i> (pIJ702) colonies.	131
Plate 4.3: Zymogram developed against L-DOPA.	133
Plate 4.4: Zymogram developed against L-DOPA.	139
Plate 4.5: Zymogram developed against L-DOPA.	140
Plate 4.6: Zymogram developed against L-DOPA.	141
Plate 5.1: Crude tyrosinase (lanes 1 and 2) developed against L-tyrosine.	172
Plate 5.2: Crude tyrosinase (lanes 1 and 2) developed against L-DOPA.	172
Plate 5.3: Crude tyrosinase (lanes 1 and 2) developed against phenol.	173
Plate 5.4: Crude tyrosinase (lanes 1 and 2) developed against p-cresol.	173
Plate 5.5: The formation of a brown precipitate above the solvent during enzyme catalysis in an aerated organic medium using crude tyrosinase.	183
Plate 5.6: A zymogram of crude tyrosinase (lanes 1 and 2) developed using L- tyrosine in chloroform.	188
Plate 5.7: A zymogram of crude tyrosinase (lanes 1 and 2) developed using L- DOPA in chloroform.	189
Plate 5.8: A zymogram developed using aqueous L-tyrosine comparing the isozyme pattern of crude (lane 4) and commercial (lane 5) tyrosinase.	190

CHAPTER 1

INTRODUCTION

PHENOLIC EFFLUENTS - TREATMENT AND DISPOSAL

Summary Aspects of phenolic wastewater treatment and disposal are reviewed, particularly focusing on the use of biological degradation systems. Polyphenol oxidase enzymes have been evaluated as a potential removal system.

In recent decades there has been a substantial increase in the manufacture of synthetic chemicals such as solvents, plasticizers, insecticides, herbicides and fungicides. More than 1 000 new compounds are marketed every year with the total annual world production of synthetic organic chemicals being over 300 million tons. The United States Environmental Protection Agency (EPA) list of priority pollutants include pesticides, halogenated aliphatics and aromatics, phenols, nitroaromatics, polychlorinated biphenyls and polycyclic aromatic hydrocarbons (Kirk and Othmer, 1982b). Of the 70 000 chemicals in commercial use, half are classified as potentially harmful to human health (WRI report, 1987). The release of these chemicals into the environment due to man's agricultural and industrial activities, often creates novel ecotoxicological problems as a result of their persistence and recalcitrance to degradation (Galli, 1990).

Many hazardous chemicals are synthesized from aliphatic and aromatic hydrocarbons. Phenolic compounds include phenol and an assortment of organic compounds containing one or more hydroxyl groups attached to an aromatic ring. In the biosphere aromatic compounds of biological origin are derivatives of the secondary metabolism of plants, biological and chemical cleavage of lignin and bioconversion of aromatic amino acids (Knoll and Winter, 1989). A variety of aromatic compounds and phenols are produced by oil and petroleum refineries, coke plants, organic chemical plants, resin and plastic manufacturers, foundries, paint-stripping operations and coal gasification and liquification plants (Lanouette, 1977; Alberti and Klibanov, 1981). The Lurgi coal gasification process produces 4-9 kg raw phenolic by-products per ton of coal processed (Schilling *et al.*, 1981), while the Sasol-II plant in South Africa annually produces 240 000 tons of phenol waste per 1.5×10^6 tons of motor fuel produced (Kirk and Othmer, 1982a). Coal conversion

wastewater contains approximately 4 900 mg.l⁻¹ phenol, 586 mg.l⁻¹ o-cresol, 1 230 mg.l⁻¹ m-cresol, 420 mg.l⁻¹ p-cresol and 320 mg.l⁻¹ dimethylphenols (Fedorak and Hrudey, 1985).

The fate of these compounds is of interest due to their toxicity to all forms of life, particularly their threat to human health. Virtually all phenolic derivatives are toxic and are considered hazardous pollutants. As little as 0.005 mg.l⁻¹ phenol imparts an objectionable taste and odour to drinking water when combined with chlorine during routine water treatment (Lanouette, 1977). Phenol is highly soluble in water, is almost tasteless in pure solution, is readily reactive with other chemicals and is rapidly absorbed by man through the gastrointestinal tract. Poisoning at high concentrations leads to corrosive local damage and subsequent systemic shock (Jarvis *et al.*, 1985). The EPA's legal limit on phenol concentration in treated drinking water is 1 µg.l⁻¹ (USA Environmental Protection Agency, 1980). A body of water contaminated with phenolic compounds may be rapidly depleted of oxygen due to high biological oxygen demands (2.4 mg O₂.mg⁻¹ phenol), presenting further pollution problems (Lanouette, 1977).

The removal of aromatic phenolic compounds from aqueous effluents therefore presents a serious problem for industries generating considerable quantities of these aromatic compounds.

Microbial activity and photolysis are the processes primarily responsible for the degradation of chlorophenols in aquatic environments. The microbial metabolism of chlorophenols involves oxidative dechlorination and hydroxylation. Ring cleavage may also occur with CO₂ as the final product. In a study carried out from 1983 to 1985 by Hwang *et al.* (1986), non-enzymatic photolysis was found to be the primary transformation process of polychlorinated phenols in surface estuarine water. For phenols and monochlorophenols, microbial degradation was the primary transformation process. Degradation rates for both processes fell in winter due to lower surface irradiance and lower water temperatures. Phenols may polymerize and form molecules very resistant to biological attack, but this also reduces the movement of chemicals into the aquatic ecosystem.

The recovery of phenolic compounds for commercial value presents another option and must be evaluated on the basis of the added value of recovered product versus the cost of

recovery. The cost of pollution control systems which would be required if recovery were not practised, should also be considered.

1.1. DEPHENOLIZATION PROCESSES

Phenolic-bearing wastes can be grouped according to strength into three categories (Lanouette, 1977):

1. High concentrations suitable for recovery and reuse or disposal by incineration;
2. Intermediate concentrations amenable to biological treatment or adsorption by activated carbon;
3. Dilute concentrations removable by activated carbon or chemical oxidation.

A range of techniques are available for the treatment of phenolic industrial effluents. These include solvent extraction, irradiation, microbial degradation, enzymatic aerobic dephenolization, adsorption on activated carbon, chemical oxidation, coagulation-flocculation, ozonation and membrane processes (Klein and Lee, 1978). Ion-exchange chromatography has been used to concentrate and separate polyphenols from high-molecular weight humus materials in natural water (Sirotkina *et al.*, 1974).

1.1.1. Physico-chemical

The Phenosolvan process, which may use diisopropyl ether (DIPE) as solvent, is a successful solvent extraction process for phenol removal (Greminger *et al.*, 1982). It utilizes a system of inert gas stripping followed by reabsorption of the stripped solvent by phenol and subsequent distillation of the mixture. Operating costs are low, but the system is complex. It is often necessary to complete extraction by activated sludge and biological oxidation processes. At Sasolburg and Secunda in South Africa, the Phenosolvan process provides tar products for the refinery and ammonia for fertilizer (Kirk and Othmer, 1982a). Although good removal efficiencies can be achieved by Phenosolvan extraction, additional and more efficient removal processes are required to meet pollution controls (Merrick, 1984).

Chem-Pro Equipment Corp. has licensed a solvent extraction process which uses methyl isobutyl ketone (MIBK) as solvent. This method utilizes a simple vacuum-steam stripping process carried out at atmospheric pressure. Operating costs are relatively high as energy has to be supplied to the stripper, but the system can be used successfully for the removal of organic substances which fall within certain ranges of volatility and solubility. Unfortunately these systems can only be used efficiently when a moderate reduction in phenol concentration is required (Greminger *et al.*, 1982).

Ultraviolet (UV) irradiation accelerates phenol oxidation when catalyzed by iron, copper or bismuth salts (Martinetz, 1986).

Adsorption on activated carbon is used for the treatment and recovery of concentrated phenolic wastes or for the "polishing" of dilute wastes for reuse or discharge. Activated carbon has a finite capacity for removing phenol. Phenol is then recovered from the carbon by chemical regeneration resulting in a concentrated phenolic stream, or by thermal regeneration which destroys the phenol. The major disadvantage is the need to regenerate large quantities of activated carbon (Lanouette, 1977).

Chemical oxidation can be used either as a polishing step or for the treatment of small volumes of concentrated waste. Hydrogen peroxide is an effective oxidizer of phenols over a wide range of temperatures and concentrations. Iron salts are used as a catalyst (Lanouette, 1977).

Oxygenation with a cuprous chloride catalyst has been used for the effective treatment of a full-strength coal-gasification wastewater at the Morgantown Energy Technology Centre. The process results in an oxidative oligomerization of phenols to form insoluble coupling products. The wastewater is first treated with lime to precipitate out the dissolved carbonate and to adjust the pH before the addition of the catalyst. The advantages of this system include simplicity, fast treatment under mild reaction conditions, the ability to withstand process interruption and fluctuations in operating parameters, and the easy disposal of organic precipitates by incineration. The process is however limited to weak phenolic waters and is sensitive to temperature, pH and nutrient loadings (Chin *et al.*, 1985).

Ozone has been used to oxidize phenols to intermediate organic compounds that are toxic, but readily biodegradable (Lanouette, 1977). The first industrial ozone wastewater application involved the oxidation of phenol and cyanide in a solution that was used to strip paint from aeroplanes. At present ozone oxidation of phenolic effluents is used in paper mills, coke mills, oil refineries and thermoplastic resin manufacturers. Phenol reacts (via pyrocatechol) by ozonolysis of the bond between the two OH-bearing carbon atoms of the aromatic ring to yield muconic acid, which is then further degraded by ozonolysis. Ozone may be used to treat water as a disinfectant - it is able to remove tastes from water, including that of chlorophenol, and eliminates phenol at a concentration of 4-6 moles ozone per mole phenol (Martinetz, 1986).

Membrane processes include reverse osmosis, ultrafiltration and electrodialysis. These processes are expensive and are not considered feasible for large-scale industrial phenolic wastewater purification (Klein and Lee, 1978).

Incineration may be used for the disposal of high concentrations of phenols (Chin *et al.*, 1985). Thermal processes are highly developed and include the following stages: drying, degassing, gasification and burning off (Martinetz, 1986).

1.1.2. Biodegradation

Conventional biological degradation systems include fluidized beds, trickling filters, packed beds, biological lagoons, oxidation ditches, rotating biological filters and activated sludge processes. Biological systems are susceptible to shock loading, long residence times are required for effective treatment and are often inadequate for the treatment of materials that are biologically very stable e.g. halogenated hydrocarbons, and for highly polluted waters (Martinetz, 1986). Advantages of biological degradation systems include low maintenance and operating costs (Worden and Donaldson, 1987). The disposal of sludge resulting from activated sludge treatments of phenolic pollutants is however expensive and amounts to approximately 50% of the total wastewater treatment costs (Verstraete and Top, 1992). Biological degradation systems are therefore most useful when used in conjunction with other dephenolization methods or when treating low- to medium-strength phenolic liquors (Dart and Stretton, 1980; Verstraete and Top, 1992). The phenolic compounds in the

wastewater are then utilized as carbon and energy sources which support the microbial community. Richards and Shieh (1989) have described industrial scale activated sludge systems where this technique may be applied.

A range of anaerobic methods exist for the treatment of phenolic wastewaters e.g. landfill wastewater and sludge co-disposal (Watson-Craik *et al.*, 1992), and packed anaerobic filters (Kuroda *et al.*, 1988). Anaerobic methods are gaining approval due to refinements such as the concentration of methanogenic biomass in the reactor and the use of on-line devices controlling the intake of toxic materials. Organic compounds are also efficiently separated from the aqueous phase in the form of biogas (Verstraete and Top, 1992).

Specific bacterial isolates which have been used widely for the treatment of pesticides and industrial effluents include *Pseudomonas* spp. for the treatment of compounds such as phenoxybenzoates (Topp and Akhtar, 1991), 2,4,6-trichlorophenol (Kiyohara *et al.*, 1992) and toluene (Inoue *et al.*, 1991), *Mycobacterium parafortuitum* E3 which degrades lower gaseous alkenes and stereospecifically produces epoxides from alkenes (Weber *et al.*, 1990), and *Desulfomonile tiedjei* DCB-1 which dehalogenates pentachlorophenol (Mohn and Kennedy, 1992). *Rhizopus arrhizus* is a filamentous fungus which has been used for the biosorption of heavy metals from polluted waters (Roux *et al.*, 1990), and immobilised *Pseudomonas* sp. and *Candida tropicalis* have been used for the degradation of phenolic waste (Munnecke, 1978).

In 1985 Chin *et al.* reported a cost analysis comparing the economic feasibility of biooxidation, solvent extraction and copper-catalyzed aerobic coupling for the dephenolization of full-strength coal-conversion wastewaters (table 1.1). Aerobic coupling is the catalyzed oxidative coupling of compounds to form insoluble products.

Table 1.1: A cost analysis of three dephenolization systems (1980 US dollar).

BIOOXIDATION (x10 ⁶)	SOLVENT EXTRACTION (x10 ⁶)	COPPER-CATALYZED AEROBIC COUPLING (x10 ⁶)
23.98	28.07	8.75

The results of this survey showed that aerobic coupling is more cost effective than other conventional dephenolization schemes. The use of microbial enzymes as biological catalysts as an alternative to copper catalysts is discussed below.

1.1.3. Enzymatic

While many of the waste-treatment processes discussed above are effective and useful, disadvantages include high cost, incompleteness of purification, formation of hazardous by-products and applicability over a limited range of concentrations (Atlow *et al.*, 1984).

Enzymatic waste treatment presents a worthwhile alternative to conventional biological degradation. Enzyme preparations for the degradation of normal household waste in grease traps and septic tanks, and the acceleration of compost heap conversions, have been on the market for quite some time (Kirk and Othmer, 1980). Peroxidase was first used for the efficient dissimilation of phenolic wastes in 1980 (Alberti and Klibanov, 1981; Atlow *et al.*, 1984). Cyanide deposits have also been successfully treated by an immobilized cyanidase enzyme in granular form (Basheer *et al.*, 1990). Rohazym ML is an enzymatic product currently being marketed in the leather and tanning industries for hydrolyzing hide offal such as fleshings and chrome shavings (Rohm and Darmstadt, 1991). Parathion hydrolase, an enzyme effective at hydrolyzing organophosphates, is produced by a variety of cultures e.g. *Pseudomonas* sp., *Flavobacterium* sp. and a recombinant *Streptomyces*. This enzyme has been successfully harnessed to treat cattle dipping liquid containing the pesticide coumaphos, and its derivative potasan, formed on the anaerobic dechlorination of coumaphos (Smith *et al.*, 1992).

Enzymes active against many classes of pesticides have also been produced in the laboratory (Munnecke, 1978). Although enzymes may be of plant, animal or microbial origin, the ubiquitous nature of microorganisms affords the opportunity to derive enzymes useful for detoxification even where microorganisms would not normally survive.

Industrial enzymes are defined as enzyme preparations manufactured for use as catalysts either directly or in the production of manufactured commodities (Kirk and Othmer, 1980). By the end of the 1980s over 2 000 enzymes had been identified with about 15% commercially available as partially purified proteins (Davies *et al.*, 1989). Major users are the food and medical industries.

Two groups of coupling enzymes for the oxidative catalysis and coupling of phenols and aromatic amines have been examined here. These include the peroxidase and polyphenol oxidase enzyme systems.

1.1.3.1. Peroxidases

Peroxidases are a complex group of haemoproteins which oxidize phenol, aromatic amines and other compounds in the presence of hydrogen peroxide (H_2O_2), alkylperoxides or aromatic peracids (Sjoblad and Bollag, 1981). Seven isoenzymes have been described; all containing ferriprotoporphyrin IX as prosthetic group. The molecular weight of the enzyme is 42 000, of which 25% is carbohydrate. The enzyme active site contains apoprotein and the haem group (The Worthington Manual, 1972).

Most higher plants are sources of this enzyme, particularly horse-radish root (*Armoracia rusticana*) from which the enzyme is commercially produced. Peroxidase is highly specific and very stable. Purified peroxidase can maintain its activity for over a year when stored at 5°C (The Worthington Manual, 1972).

Peroxidase plays an important role in the natural degradation of lignin. The first lignin-degrading enzyme, ligninase, was discovered in late 1982 and is produced by *Phanerochaete chrysosporium* (Tien and Kirk, 1984; Evans and Fuchs, 1988). *P. chrysosporium* is a white-rot fungus which produces an extracellular enzyme that

degrades lignin in the presence of hydrogen peroxide. This enzyme is now known to be a type of peroxidase. The peroxidase reacts with H_2O_2 to produce an oxo-Fe(IV)-porphyrin cation radical. This highly reactive species removes an electron from one of lignin's many oxygenated aryl rings, forming an aromatic cation radical. These radicals cause side-chain fragmentation (C-C cleavage), which leads to breakdown of the lignin polymer (Evans and Fuchs, 1988).

Alberti and Klivanov (1981) first proposed the use of horseradish peroxidase for the removal of phenols from aqueous wastewaters. In 1984 Atlow *et al.* used peroxidases to remove various phenols, aromatic amines, hydrocarbons and polychlorinated biphenyls (PCBs) from effluent. This process was highly successful and showed a 85%-90% removal efficiency using 25 units.l⁻¹ peroxidase and 2mM H_2O_2 . A disadvantage of peroxidase-catalyzed dephenolization is the use of stoichiometric amounts of costly hydrogen peroxide as oxidant.

1.1.3.2. Polyphenol oxidases

As polyphenol oxidases were identified over 100 years ago (Scott, 1975), an examination of this enzyme system requires a thorough review of research carried out over the years.

Polyphenol oxidase, in contrast to peroxidase, utilizes molecular oxygen as oxidant. These enzymes are ubiquitous in nature and are involved in the biosynthesis of a wide range of natural products including lignins, tannins, melanins, antibiotics and alkaloids, as well as the polymerization of naturally-occurring phenols resulting in the formation of humic acid and humus in the soil (Randtke and Larson, 1984). These reactions are carried out by an oxidative coupling process. Once the enzymes have become stabilized in the soil they manifest resistance to humidity, temperature and environmental changes (Kiss *et al.*, 1975; Skuijns, 1976).

Oxidative coupling is a process by which phenolic compounds or aromatic amines are linked together after oxidation by an enzyme or a suitable chemical reagent. The initial

step in the reaction is the production of an aryloxy radical from phenol by the removal of an electron and hydrogen ion (H^+) from the hydroxyl group.

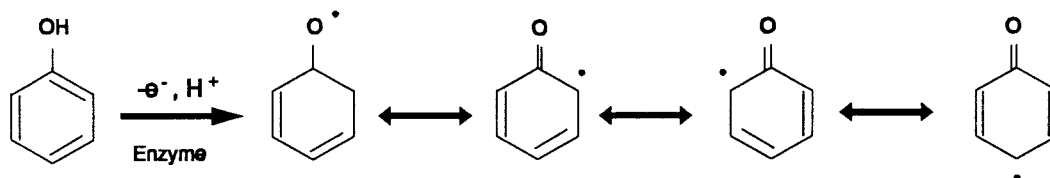


Figure 1.1: The formation of aryloxy radicals from phenol (Sjogblad and Bollag, 1981).

These radicals are stable at room temperature and diffuse from the active site of the enzyme into solution. Two free radicals can couple at positions ortho- and para- to the hydroxyl group and form dimers. These C-C coupled dimers can be oxidized further to extended quinones which spontaneously polymerize. The polymerized polyaromatic products are water-insoluble and can be removed by simple methods such as filtration or sedimentation (Sjogblad and Bollag, 1981). The enzyme system can also polymerize polyhydroxy aromatic compounds. Intermediates may be resonance stabilized on the large condensed ring network, particularly semi-quinone radicals from dihydric phenolic substrates (Suflita, 1980).

Pollutants easily removed from wastewaters may aid in the precipitation of other phenols and aromatic amines with low removal efficiencies. Free radicals enzymatically produced from compounds with high removal efficiencies interact with those from products of low removal efficiency. A high molecular weight mixed polymer is formed which readily precipitates. These free radicals may therefore be used to remove even non-phenolic and non-aromatic amine compounds (Sjogblad and Bollag, 1981).

Polyphenol oxidases are metal-containing enzymes that catalyze the oxidation of mono- and o-dihydroxyphenolic substances. There has been considerable confusion concerning enzyme nomenclature as enzymes often exhibit similar substrate specificities. In addition to polyphenol oxidase, the enzyme system is also known as tyrosinase, polyphenolase,

phenolase, catechol oxidase, cresolase and catecholase. The following terms may be regarded as synonymous for the purposes of this study: polyphenol oxidase, tyrosinase, polyphenolase, phenolase.

Two distinct enzyme groups exist i.e. the tyrosinases and laccases. Both enzymes contain Cu^{2+} and require molecular oxygen, but no coenzyme, for activity. They catalyze the consumption of one molecule of oxygen per molecule of substrate, one oxygen atom appearing in the substrate and the other undergoing reduction (Matthew and Parpia, 1971). The enzymes are inhibited by substances forming stable complexes with copper e.g. KCN, sodium azide, H_2S and diethylidithiocarbamate. They appear to differ with respect to physiochemical properties in the reactions they catalyze and in their substrate specificities, but may yield the same coupled products from phenolic substrates. Tyrosinase appears to be more widely distributed than laccase (Sjogblad and Bollag, 1981).

In 1957 Bonner reviewed the hypotheses that had been advanced to explain the behaviour of the enzyme system (references cited in Bonner, 1957). The long history of the enzyme system is clearly shown by this overview.

In 1928 Onslow and Robinson first proposed that the oxidation of phenolic compounds was limited to o-dihydroxyphenols, the monophenol being converted to an o-dihydroxyphenol by hydrogen peroxide resulting from the o-dihydroxyphenol oxidation, and not by the enzyme complex. Dawson and Ludwig (1938) showed that hydrogen peroxide was not formed during o-dihydroxyphenol oxidation.

Richter (1934) stated that two separate enzymes exist. One for the oxidation of monophenols and another for that of o-dihydroxyphenols. This theory was rejected by Mallette and Dawson in 1949.

In 1938 Keilin and Mann reported that monophenol oxidation requires a heat stable activator. There was no real support for this theory.

Kertesz (1939, 1951 and 1952) stated that the conversion of monophenol to o-dihydroxyphenol was catalyzed by free and nonprotein-bound metallic ions e.g. Cu, Co, Ni and Va.

In 1955 Mason *et al.* proved that monophenols are enzymatically degraded to o-dihydroxyphenols. Dawson *et al.* (1951), cited in Bonner (1957), reported that mono- and o-dihydroxyphenol oxidation is carried out by the same enzyme (tyrosinase) and the monophenol oxidizing ability is lost due to enzyme fragmentation during purification.

Bonner (1957) proposed that a close relationship exists between phenol oxidase (tyrosinase) and laccase. The association of these enzymes in many fungi has led to the suggestion that there is an interconversion of one enzyme form into the other, the difference in specificity being accounted for by the substrate and the presence or absence of co-factors. This theory supports the identical nature of the enzymes.

Similarities in tyrosinase and laccase production by *Neurospora crassa* indicates common control (Froehner and Eriksson, 1974). Tyrosinase and laccase have also been found together during morphogenesis of sclerotia of *Sclerotinia sclerotiorum*, but were isolated at different stages of sclerotial development. Laccase was present throughout development, whereas tyrosinase was only present in sclerotia. Although enzyme production overlapped, two distinct enzymes with different substrates and functions were present (Wong and Willetts, 1974).

Laccase and tyrosinase are characterized by their different pH optima, temperature sensitivities, inhibitors and substrate specificities (table 1.2). Tyrosinase is produced by white and brown rot fungi, whereas laccase is specific for white rots. White rots decompose both cellulose and lignin, whereas brown rots degrade cellulose only (Blaich and Esser, 1975; R  ih   and Sundman, 1975). As laccase and tyrosinase occur together in many natural environments, it is necessary to identify the enzymes correctly.

Table 1.2: Laccase and tyrosinase substrate specificities (adapted from Käärik, 1965; Sjöblad and Bollag, 1981).

SUBSTRATE	ENZYME	
	LACCASE	TYROSINASE
monophenols	-	oxidized
o-dihydroxyphenol	oxidized	oxidized
p-dihydroxyphenol	oxidized	-
m-dihydroxyphenol	-	-

Table 1.3 shows several characteristics used to differentiate laccase, tyrosinase and peroxidase.

Table 1.3: Characteristics of laccase, tyrosinase and peroxidase enzymes (Sjöblad and Bollag, 1981).

CHARACTERISTIC	LACCASE	TYROSINASE	PEROXIDASE
Presence of Cu	+	+	-
Presence of Fe	-	-	+
Inhibition by CO	-	+	-
Occurrence of hydroxylation	-	+	- (+)
Absorption spectra peak at 280nm	+	+	
Absorption spectra peak at 615nm	+	-	
H ₂ O ₂ requirement	-	-	+

Laccase

This enzyme was first detected in the Japanese lacquer tree, *Rhus vernicifera*, by Yoshida in 1883 (Nord and Schubert, 1967). Other sources include cabbages, turnips, apples, asparagus, potatoes and mushrooms. Plant laccases oxidize o- and p- substituted diphenols. The known distribution of laccase in higher plants is limited. Fungal laccase oxidizes o- and p-diphenols, amino phenols and diaminoaromatic compounds using molecular oxygen and has a wider substrate specificity than plant laccases (Pickard and Westlake, 1970). Laccases from different sources therefore form different products at different pH values and on different media, but all enzymes generate the same chemicals at a particular pH (Bollag and Leonowicz, 1984).

Laccase is generally extracellular and extruded into the medium. However, Blaich and Esser (1975) have reported the discovery of an intracellular enzyme. Very little laccase is extruded into the growth culture of *Podospora anserina* (Molitoris and Esser, 1971).

Although laccase and tyrosinase are generally produced by different organisms, Horowitz and Fling (1953) (cited in Froehner and Eriksson, 1974) first reported the presence of laccase in the tyrosinase-producing fungus, *Neurospora crassa*. Laccase was produced only in stationary liquid culture and induced by protein synthesis inhibitors such as cycloheximide and actinomycin D. Laccase is depressed by an unstable, continuously synthesized protein repressor in rapidly growing cultures. When the concentration of the repressor is reduced by protein synthesis inhibition, the enzyme is derepressed (Froehner and Eriksson, 1974).

Most fungal laccases exhibit maximal activity in the pH range 4 - 5.5. A number of compounds have been reported which induce enzyme synthesis, e.g. 2,5-xylydine, cycloheximide, ferulic acid and guaiacol, but *de novo* synthesis has been reported for the fungus *Pleurotus ostreatus* (Sjogblad and Bollag, 1981). The choice of inducer seems to depend on the source of the enzyme. The molecular weight, copper content, enzyme induction or synthesis and substrate and pH specificity also depends on the enzyme source. The activity of some laccase isoenzymes may be enhanced by the addition of H₂O₂;

differentiation between peroxidase and laccase on the basis of substrate transformation is therefore difficult (Blaich and Esser, 1975).

Laccase contains tightly bound copper, but was earlier suspected of being a manganese enzyme. The manganese found in the crude extract disappears on purification, whereas the copper content increases. Laccase is a deep blue colour which appears to be due to the copper bound within the molecule. This blue colour disappears reversibly upon addition of the substrate and is caused by a copper protein named stellacyanin, which has little or no oxidase activity and is high in carbohydrate content. In the oxidised enzyme the copper is present in the cupric state. Enzyme activity parallels the content of the cupric form and not that of total copper (Levine, 1966).

There is some controversy as to the molecular weight and copper content of this enzyme. Molecular weights ranging from 64 000 in *Polyporus versicolor*, 110 000 - 140 000 in the lacquer tree, 117 000 in *Aspergillus nidulans* and 144 000 in higher plants have been reported (Wood, 1980; Ishigami and Yamada, 1986). Sjöblad and Bollag (1981) reported laccase molecular weight to be 120 000, with each molecule containing four atoms of copper. Wood (1980) confirmed this molecular weight for laccase extracted from *Agaricus bisporus*, but stated that the copper content was two atoms per molecule. Electron-spin resonance (ESR) studies showed that although the copper content was four atoms per molecule, only two were in the cuprous form (Mosbach, 1963).

Multiple forms of this enzyme exist. A number of researchers have reported the isolation of two fractions - laccase A and B - from *P. versicolor* (Fahraeus and Reinhammar, 1967; Pickard and Westlake, 1970; Leonowicz *et al.*, 1978; Fagan and Fergus, 1984). Fahraeus and Reinhammar (1967) reported a molecular weight of 64 000 for laccase A. Mosbach (1963) reported that both forms A and B were present in the culture medium and therefore proposes that the fungus synthesizes two separate laccases.

The production of three laccases by *Podospora anserina* has been reported; the particular component and proportion of enzyme produced being dependent on the age of the mycelia. The substrate specificities of the components differ (Molitoris and Esser, 1971; Schanel and Esser, 1971).

Leonowicz *et al.* (1978) stated that both inducible and constitutive forms of the enzyme exist in the Basidiomycetes; both oxidizing o- and p-diphenols more actively than m-diphenols. Induction of the enzyme was preceded by the *de novo* appearance of a new mRNA species specific for the inducible form (Leonowicz *et al.*, 1978; Leonowicz and Trojanowski, 1978).

Five enzymes fractions have been isolated from *Pleurotus ostreatus* (Leonowicz *et al.*, 1978). The choice of separation system affects results when determining enzyme structure. Laccase extracted from the mycelia of *A. bisporus* showed a single enzymically active electrophoretic band at pH 4.5, but several at pH 8.9 (Wood, 1980). Laccase isoenzymes show a qualitative variability with respect to substrate specificities.

Multiple electrophoretic forms of other fungal polyphenol oxidases have been shown to be due to small differences in the length and properties of the glycan chains on sub-fractions of the enzyme population (Esser and Minuth, 1971).

The amino acids present in low amounts in laccase e.g. tryptophan, lysine and methionine, are all present in multiples of two. It is therefore tempting to assume that the enzyme might consist of two identical subunits (Fahraeus and Reinhammar, 1967). It is however evident that laccase is not a single entity but rather represents a group of closely related enzymes that can be distinguished from each other.

Laccase has many functions. It plays a role in lignin degradation and has been implicated in the pigmentation of fungal spores and the development of spore-bearing structures (De Vries *et al.*, 1986). It is produced by a number of wood-inhabiting Basidiomycetes and seems essential for the degradation and subsequent metabolism of lignin by these organisms. Lignin, a polymer of one or more phenylpropane monomers, is mobilized as an energy source (Ander and Eriksson, 1977). Another possible role of the enzyme in nature is to solubilize lignin and make it more susceptible to other enzymes e.g. laccase from *P. versicolor* grown in submerged culture (Dion, 1952).

Until 1977 only two kinds of enzymes involved in lignin biodegradation had been described i.e. phenol oxidases (intra- and extracellular) and peroxidases. Lignin

degradation appeared to be favoured by the presence of cellulose and stimulated by the degree of polymerization of the cellulose. This is due to the presence of the enzyme cellobiose:quinone oxidoreductase, discovered as late as 1974. The enzyme complex is induced in media containing cellulose and oxidizes cellobiose to cellobionolactone. It facilitates lignin degradation by preventing polymerization reactions catalyzed by laccase. Quinones, produced by laccase, are used as hydrogen receptors. Quinones and phenol radicals are reduced to corresponding phenols. These phenols, especially catechol moieties, may be readily utilized by other enzymes and further metabolized (Szajer and Targonski, 1977). Oxidative polymerization therefore does not seem to take place in natural wood as the initial products of laccase action are immediately reduced to the phenolic state (Haars *et al.*, 1981).

Protection gained by laccase production or presence has been widely detected. The darkening and hardening of lacquer tree latex is a result of the action of laccase on these substances and may indicate a protective function (Levine, 1966).

Laccase appears to be produced during the encystation of an amoeboid organism, *Acanthamoeba castellanii* (Sykes and Band, 1985). The melanized cyst wall is resistant to microbial degradation and to physical and chemical agents. As quinones and quinone-tanned proteins (products of the laccase-catalyzed enzyme reaction) are toxic they form a defence mechanism (Sykes and Band, 1985).

In 1983 Bollag proved that fungal laccases can oxidatively couple phenolic compounds (which are derivatives of humic substances) together with other chemical compounds. Syringic and vanillic acids are phenolic carboxylic acids (transformed by laccase) that contribute to the formation of humic substances in the soil and appear as intermediates in the biotransformation of lignin by white-rot fungi (Leonowicz *et al.*, 1984). Results support the assumption that laccases are involved in the formation of humus and the incorporation of organic xenobiotics into soil organic matter. Laccase substrates do occur in natural soils, but the enzyme is not easily extracted. Laccase may therefore be immobilized or bound in native soils (Leonowicz and Bollag, 1987).

Laccase's ability to detoxify or alter the toxicity of phenolic pollutants may be due to its capacity to transform or cross-couple the pollutants with phenols, humic acids, clays and other materials (Bollag *et al.*, 1988).

Tyrosinase

Tyrosinase is also known as polyphenol oxidase, DOPA oxidase, phenolase and catechol oxidase. It is a cuprous protein which has been known since 1896 and is responsible for the formation of the brown, red and tan pigments - melanins - which occur in the skin, eyes and feathers of animals throughout the phylogenetic scale (Mason, 1966).

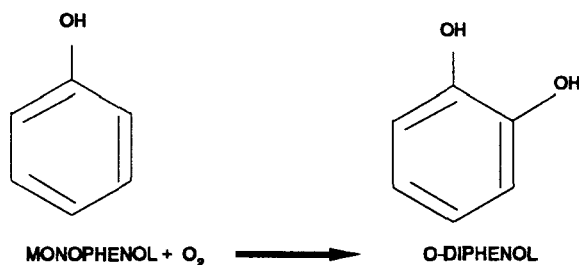
Tyrosinase was originally named because of its ability to catalyse the aerobic oxidation of the monophenolic amino acid, tyrosine. It is the only enzyme known to catalyse the direct aerobic oxidation of monophenols. In higher plants and fungi the enzyme is fairly non-specific and oxidizes many mono- and o-dihydroxyphenolic substances. Its sources include mushrooms, potato tubers, apples, bananas, avocados, tea leaves, coffee beans and tobacco leaves. In plants oxidative enzymes may contribute to the defence of the host against infection by invading yeasts, fungal spores, enteric bacteria and streptococci. The enzymatic production of toxic quinones from phenols may be responsible for the increased bactericidal effect (Scott, 1975).

The pH optimum for enzyme activity is between pH 5 and 7. At higher pH values autooxidation of the substrate takes place and below pH 3 enzyme activity is lost and cannot be restored (Scott, 1975).

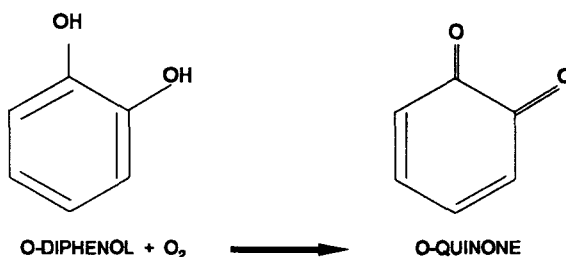
The copper content of *A. bisporus* tyrosinase varies from 0.16% - 0.26%. The molecular weight of purified protein containing 4 copper atoms per mole is approximately 100 000 (Sjoblod and Bollag, 1981). The highest molecular weight tyrosinase from fungi is produced by *Aspergillus nidulans* (Bull and Carter, 1973).

Tyrosinase appears to be bifunctional and catalyses the following two reactions in aqueous systems:

1. Cresolase reaction.



2. Catecholase reaction.



The final stage in the phenol-oxidising reaction is the non-enzymatic oxidative polymerisation of o-quinones to water-insoluble pigments or polyphenolic products (Scott, 1975; Sjobland and Bollag, 1981).

Melanins are formed by the oxidative polymerization of phenolic compounds such as tyrosine, catechol and 1,8-dihydroxynaphthalene (allomelanins), DOPA (eumelanins) and cysteinyl-DOPA (phaeomelanins). The eumelanins and phaeomelanins are found mainly in animal species, whereas the allomelanins are found in plants and microorganisms. Melanin-producers amongst microorganisms include *Micrococcus*, *Bacillus*, *Streptomyces*, *Vibrio*, *Mycobacterium* and *Rhizobium* species. Melanins are not considered essential for

growth and development of cells, but do play a protective role e.g. in the presence of UV radiation. They act as UV absorbers, amorphous semiconductors and novel biopolymers with drug-binding characteristics (della-Cioppa *et al.*, 1990). Melanins also act as cation exchange polymers which can protect tissues against oxidizing and reducing conditions and trap free radicals which may damage the cell (Shivprasad and Page, 1989).

On enzyme purification, the cresolase activity diminishes in relation to the catecholase activity as it is less stable. Denaturation will therefore lead to an increase in the catechol/cresol (cat/cre) ratio. With a monophenol as substrate, there does appear to be a characteristic induction period - this is eliminated by the addition of a trace of catechol (Vanni and Gastaldi, 1990). The enzyme must therefore be "primed" or "activated" by a reducing agent such as ascorbic acid or hydroquinone before initiating the cresolase activity (Matthew and Parpia, 1971). Oxidizing agents therefore lengthen the induction period and reducing agents markedly shorten it.

In 1975 McIntyre and Vaughan (cited in Jacobsohn *et al.*, 1988) proposed the existence of three substrate binding sites for the beet enzyme. One for the o-dihydroxyphenol activating the hydroxylation reaction, the second for the monohydric substrate, and the third for the o-dihydroxyphenol functioning as substrate for the formation of the quinone (Jacobsohn *et al.*, 1988).

In 1960 Dressler and Dawson proposed that two different activity centres may exist on the tyrosinase enzyme, one each for the two types of catalytic ability (cited in Brooks and Dawson, 1966). According to Kertesz and Zito (1957) there do not appear to be two enzymes present as tyrosinase can be obtained in a homogeneous form on ultracentrifugation and electrophoresis. Both activities are also inhibited to the same extent by inhibitors and by copper removal.

Tyrosinase has been identified in almost half the *Streptomyces* isolated from natural habitats. Although the Streptomyces are a promising source of tyrosinase, the function of the enzyme in these organisms is still unknown (Held and Kutzner, 1990).

Tyrosinase is a common enzyme in the mycelia and fruiting bodies of several fungi, including litter-decomposing, coprophilic and mycorrhiza-forming hymenomycetes. Ramstedt and Söderhall (1983) proposed that phenol derivatives may be produced in host cells as a response to mycorrhizal penetration. Low enzyme activities could therefore be an adaptation to a symbiotic life, where the symbiont must avoid eliciting host resistance reactions by the excretion of enzymes.

Tyrosinase is found mainly in the mycelia of brown-rot fungi, but white rot sources have been identified e.g. *Polyporous dichrous*, which produces tyrosinase on lignosulphate-containing agar upon ageing (Räihä and Sundman, 1975). In contrast to laccase, tyrosinase is a typical endoenzyme (Bull and Carter, 1973). In some fungi tyrosinase is only present under special cultural conditions (Lyr and Luthardt, 1965).

In sodium-dependent *Azotobacter chroococcum*, a free-living nitrogen-fixing obligate aerobe, polyphenol oxidase was produced under high aeration and nitrogen-fixing conditions. This resulted in the production of a cell-associated black pigment, namely catechol melanin. Melanin formation appears to serve as an oxygen protection system; important for aerobic growth during nitrogen fixation. Iron and copper are important transition metal ions needed for the superoxide-dependent formation of hydroxyl radicals. Molecules binding iron, e.g. catechol, can prevent it from participating in the Fenton reaction and therefore protect a cell from the damaging effects of the hydroxyl radical. Melanins will also bind cations and prevent membrane damage by hydroxyl radicals. Catechol and melanin formation may have evolved as a chemical protection system for aerobic nitrogen fixation (Shivprasad and Page, 1989).

Large quantities of enzyme are induced in *N. crassa* by starvation in pH 6.0 phosphate buffer or by the addition of certain toxic substances e.g. amino acid analogues. Gutteridge and Robb (1975) showed that the enzyme can be induced by adding sucrose and cycloheximide. Induction involves a *de novo* synthesis of the enzyme (Horowitz, 1965). Fox and Burnett (1958) reported that crude extracts contain protyrosinase which undergo enzymatic activation during incubation, even in the absence of substrate. The shortening of the induction period is largely due to the conversion of protyrosinase to tyrosinase. DOPA is slowly produced from small amounts of precursors in the extract and may act as

a catalyst converting protyrosinase to tyrosinase. Figure 1.2 represents the oxidation of tyrosine by the tyrosinase of *N. crassa*.

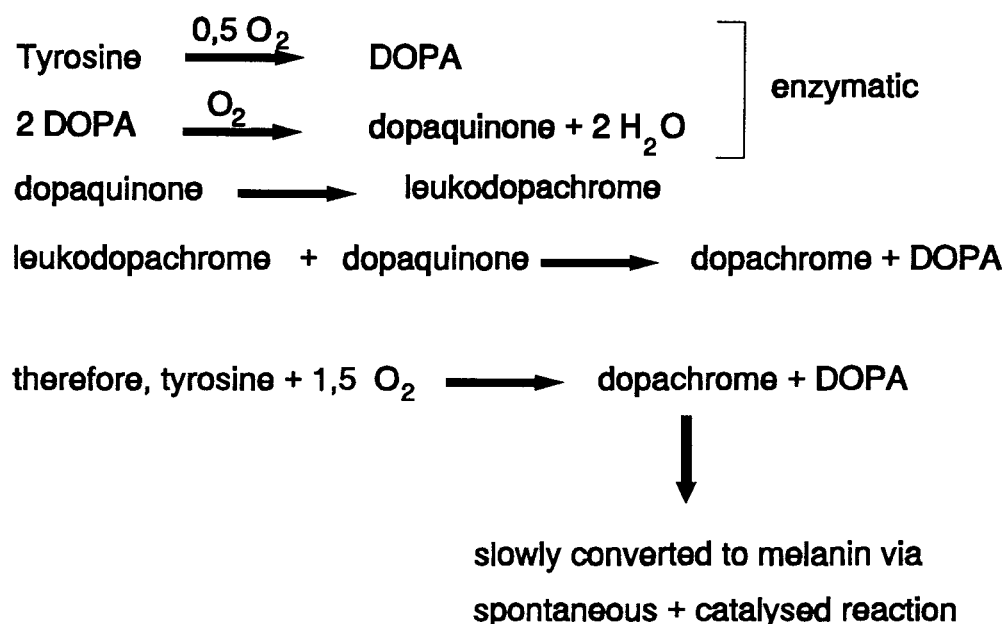


Figure 1.2: Tyrosine oxidation by *N. crassa* tyrosinase (Horowitz *et al.*, 1970).

The presence of an inactive pre-tyrosinase in the haemolymph of the silkworm, *Bombyx mori*, has been reported by Ashida and Ohnishi (1967). On homogenization the pre-tyrosinase is activated by a proteinaceous endogenous activator present in the cuticle. A non-protein factor also appears to be present.

Mammalian tyrosinase (purified from hamster melanoma) has limited oxidizing ability (Scott, 1975) and catalyses tyrosine to melanin via L-DOPA, dopaquinone and dopachrome intermediates (Pomerantz and Peh-Chen Li, 1970).

In the first step of melanogenesis i.e. the hydroxylation of L-tyrosine to L-DOPA, L-DOPA may function as a cofactor and only as a substrate in the next stage of the reaction. Melanin is formed by the polymerization of indole-5,6-quinone, a metabolite from tyrosine or DOPA, by repeated oxidative condensations involving the anionoid centre of the pyrrole

ring of one molecule and the cationoid centre of the benzonoid part of another. DOPA or its intermediates may easily be attracted by melanin or a melanin precursor for copolymerization. Tyrosine may also be incorporated into melanin by polymerization and copolymerization. The more highly oxidized products are chemically unstable and may polymerize to higher homologs without the further intervention of enzymes. Electron-spin resonance (ESR) studies have shown the irregularity of the melanin polymer (Chen and Chavin, 1966).

Melanin production requires the translocation of mammalian tyrosinase from the trans-Golgi-reticulum (TGR) to the melanosomes and is then restricted to these organelles. As melanin is potentially toxic to pigment cells due to the production of free radicals and toxic products of melanogenesis, it is kept under the strict control of multiple gene products (Slominski and Constantino, 1991). These authors also proposed that either L-DOPA or its oxidation products stimulate intracellular tyrosinase concentrations and regulate tyrosinase mRNA levels. The addition of L-DOPA to hamster amelanotic melanoma cells resulted in an increase in cell pigmentation, tyrosinase concentration and activity.

Mammalian tyrosinase has been shown to occur in a number of forms separable on electrophoresis (Holstein *et al.*, 1967).

As tyrosinase is translocated to the melanosomes, it is important to evaluate and categorize the effect of tyrosinase on cellular proteins. Cory and Frieden (1967) showed that tyrosinase does catalyze the oxidation of tyrosyl residues of some intact proteins; often causing partial or complete loss of biological activity.

Mushroom tyrosinase has been implicated in the production of catechol oestrogens. It reacts with steroid oestrogens to produce 2-hydroxylated derivatives. This property has been utilized to develop methods for the synthesis of catechol oestrogens. These oestrogenic compounds are naturally occurring and biologically active. They are formed by aromatic hydroxylations in mammalian tissues (Woerdenbag *et al.*, 1990), and have been shown to be synthesized by human breast cancer tissue *in vitro* in the presence of an organic peroxide (Jacobsohn *et al.*, 1988). Their availability as reference compounds for analytical and pharmacological use is therefore important.

17 β -estradiol (a phenolic steroid) complexed with β -cyclodextrin, is o-hydroxylated by a phenoloxidase extracted from a *Mucuna pruriens* cell culture to produce 4-hydroxylestradiol, a catechol oestrogen. Sodium ascorbate serves as cofactor and anti-oxidant to prevent quinone formation. The efficiency and product yield of such a reaction is limited by the water solubility of the substrate. The substrate is therefore complexed with β -cyclodextrin and a soluble inclusion complex is formed. Cyclodextrins are used in plant cell biotechnology for their solubilizing action as they are not broken down or used as a carbon source. Polyphenol oxidase extracted from *M. pruriens*, a member of the Spermatophyta, converts the substrate regiospecifically (Woerdenbag *et al.*, 1990).

There appears to be a physiological role in primary metabolism for the tyrosinase produced by *A. nidulans* i.e. it plays a role in reoxidizing extramitochondrial reduced dinucleotide coenzymes. This theory is supported by the following factors (Bull and Carter, 1973):

1. Tyrosinase occurs in both particulate and soluble cell fractions;
2. Tyrosinase has a low affinity for oxygen and activation is therefore likely when cellular oxygen levels increase, e.g. when mitochondrial respiration is repressed at high dilution rates;
3. The oxygen uptake rate of whole mycelia is depressed by the tyrosine analogue DL- β -phenyl lactic acid.

An endogenous tyrosinase inhibitor which causes non-competitive inhibition of the enzyme has been isolated. This could be a safety mechanism for the cell which traps any tyrosinase entering the cytoplasm, therefore preventing indiscriminate melanin formation in the cytoplasm (Bull and Carter, 1973).

The biochemical characteristics of tyrosinase

The absorption spectrum of mushroom polyphenol oxidase shows a very intense and sharp band with a maximum around 280nm and a low and diffuse shoulder around 340nm (Kertesz and Zito, 1957; Kertesz, 1966). This is due to the high aromatic amino acid content of tyrosinase i.e. 10 tyrosine residues and 11 tryptophan residues for a minimal molecular weight of 31 800, corresponding to one copper atom per molecule. Gutteridge

and Robb (1975) reported the evidence of a small peak at 345nm for tyrosinase extracted from *N. crassa*. This appeared to be oxytyrosinase present in the freshly prepared enzyme.

A number of researchers have reported the isolation of multiple forms of the phenolase enzyme. Smith and Krueger (1962) detected five mushroom tyrosinase activities on hydroxylapatite chromatographic columns. In 1963 Bouchilloux *et al.* isolated four proteins (the three latter proteins in homogeneous form): α , β , γ and σ -tyrosinases. This study showed that subunits are involved in tyrosinase structure i.e. tyrosinase is tetrameric. A molecular weight of 34 500 had been reported for mushroom tyrosinase, but a subunit containing one atom of copper per molecule has a minimal molecular weight of approximately 30 000. Thus four subunits together would contain four atoms of copper with a total molecular weight of approximately 100 000 - 120 000. A very soluble tyrosinase may exist, being Smith and Krueger's fifth tyrosinase (Bouchilloux *et al.*, 1963).

In mushrooms, some of these forms (isoenzymes/isozymes) have been found to be interconvertible depending on conditions of pH, ionic strength and protein concentration (Jolley and Mason, 1965; Matthew and Parpia, 1971). Smith and Krueger (1962) used calcium phosphate gel adsorption techniques and column chromatography to prove that the occurrence of various enzymes in the mushroom was not due to fragmentation of an original single enzyme entity. All enzyme types were shown to occur naturally.

There are a number of researchers that refute enzyme multiplicity. Kertesz and Zito isolated a single tyrosinase enzyme of molecular weight 120 kDa from mushrooms, and proposed that multiple forms found by other researchers were artifacts of preparation or minor components of low activity (Jacobsohn *et al.*, 1988). Jolley *et al.* (1969) isolated four isoenzymes of which they claimed two to be conformational isomers; one to be a quinone-bound form of one of the former and the fourth to be a true isoenzyme.

Tyrosinase extracted from *A. nidulans* is also heterogeneous and has two fractions or components i.e. E_1 and E_2 . Four bands (I-IV) were seen on PAGE; probably indicating heterogeneity at the tertiary protein structure level. The enzyme exists in a monomer (molecular weight: 1.3×10^5) - tetramer (molecular weight: 5.2×10^5) equilibrium. Heterogeneity is due to association (Bull and Carter, 1973).

Purified tyrosinase extracted from *N. crassa* is homogeneous on ultracentrifugation and electrophoresis. There is no evidence that dimers or tetramers are necessary for activity. The enzyme is active as a monomer (molecular weight 33 000) (Gutteridge and Robb, 1975). Aggregation to a tetramer (molecular weight 120 000) occurs readily and reversibly (Horowitz *et al.*, 1970). This claim was disputed by Gutteridge and Robb (1975) who stated that there was no evidence for a quaternary complex. Several allelic forms of the enzyme, differing in thermostability and electrophoretic mobility, have been described (Horowitz, 1956; Horowitz *et al.*, 1970). Four were identified by Fling *et al.* (1963); two known as S and L-tyrosinases respectively.

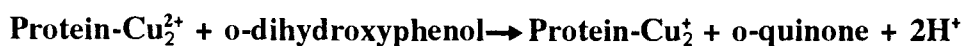
The role of copper in tyrosinase

In 1939 Kubowitz (cited in Karkharis and Friedin, 1961) established that tyrosinase contained copper essential for activity. He proved that activity was lost on copper removal and regained once the copper was replaced. Even under inhibition conditions, mushroom tyrosinase was converted to a fully active enzyme by the addition of cupric ions.

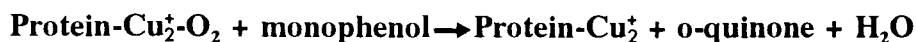
Copper in the active polyphenolase enzyme is in the cuprous or monovalent form. Cupric copper added to the apoenzyme is immediately reduced to the active cuprous form; copper is therefore lost from the enzyme moiety in the functioning enzyme system. In 1956 Mason stated that two copper atoms in the enzyme were reduced to the cuprous form in the presence of one molecule of o-dihydroxyphenol. From one molecule of oxygen, one atom is consumed during the hydroxylation reaction of monophenols, while another is converted to water, requiring two electrons supplied by the cuprous atoms. One molecule of oxygen therefore oxidizes two molecules of reducing substrate in reactions catalyzed by tyrosinase (as shown below). It has been suggested that a quaternary complex of enzyme, oxygen and two molecules of substrate is formed during the oxidation of substrate by tyrosinase (Matthew and Parpia, 1971).



The copper is now in the inactive (cupric) form, which requires reactivation. This is accomplished by the oxidation of dihydroxyphenol to quinone.



The three steps are believed to occur in one stage, which can be represented as follows:



The ability of the oxygen complex to act as a centre of hydroxylation depends upon the orientation of the electrons around the complex, and this then depends upon the position of the two copper atoms. In 1956 Mason suggested the following three positions; option C i.e. asymmetric with coordinated oxygen atom, being preferred.

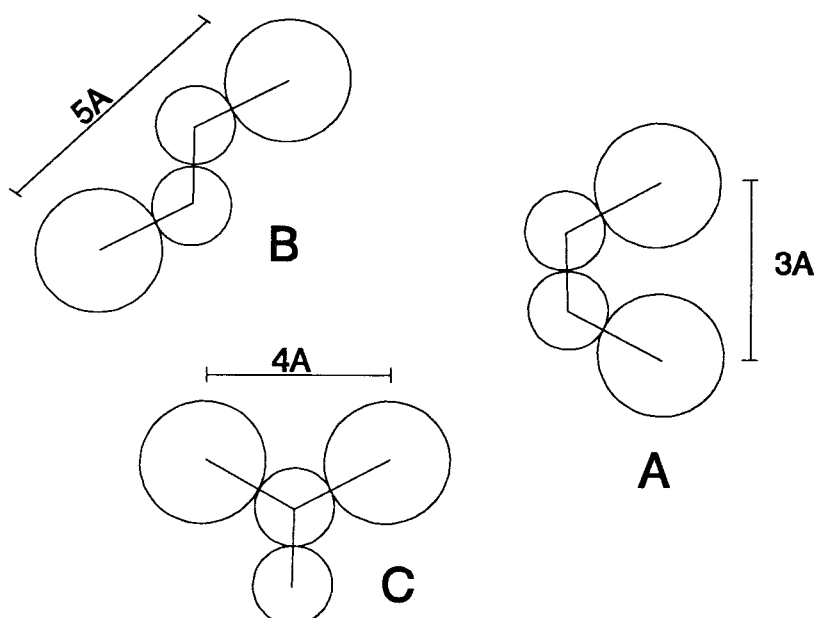


Figure 1.3: Scale drawings depicting configurations of the cresolase-active copper-oxygen complex. The large circles represent copper atoms, the small circles oxygen (Matthew and Parpia, 1971).

This research disclosed the presence of at least two distinct substrate binding sites on the enzyme, one with a high affinity for aromatic compounds including phenolic substrates, the other for copper and oxygen. For *N. crassa* tyrosinase, the most likely site of copper attachment is reported to be at two of six histidine residues (Fling *et al.*, 1963). This

theory is supported by *Streptomyces glaucescens* tyrosinase copper binding to histidine residues in the enzyme active centre (Held and Kutzner, 1990).

Any disturbance of the protein structure or amino acid side chains involved in the attachment of copper atoms during extraction and purification affects the cresolase activity, but not the catecholase activity, which is not as dependent on the attachment of the two copper atoms (Matthew and Parpia, 1971).

In 1967 Harrison *et al.* reported a study on stereospecificity in mushroom tyrosinase-catalyzed oxidation reactions using fluorescence spectrometry. The observed effects of side chain substituents on oxidation velocities using adrenaline, noradrenaline and L-DOPA as substrates, suggested that tyrosinase contains a stereospecific reaction rate control site. It was proposed that substrate side chains bind to the control site through chelation with copper and function as reaction rate modulators. This may explain the striking differences apparent between tyrosinases from different sources e.g. catechol is a very good substrate for mushroom tyrosinase, but unsubstituted catechol is a poor substrate for mammalian tyrosinase. Substrate specificities also differ depending on their side chains, the number of side chains and orientation. It was thought that these differences might be attributed to the presence of a subsite at which rate-determining interactions involving the side chain groupings occur. Interactions between side chains and an enzymic site which is in close proximity to, but separate from the site of interaction with the catecholic group, may therefore take place. A three-point attachment may be required for a stereospecific reaction, one attachment with the catecholic group and two with the side chain. When reacting with unsubstituted catechol, the side chain site is unoccupied and would exert no effect on oxidation velocity, whereas substituted catechol would modulate the reaction rate via interaction with the control site of the enzyme. Previously the effect of side chains was attributed to a general blocking effect at the active site, but Harrison *et al.* (1967) have shown that the rate is controlled by an extremely specific interaction occurring at the site at which the side chain is bound.

In conclusion, the copper contained in the tyrosinase enzyme has been shown to be indispensable for its activity and is thought to be located in the catalytic site of the enzyme, participating in the electron transfer processes in the binding and activation of

oxygen and the binding of substrate. It is uniquely suited for this role due to its oxygen-binding and activation functions and its ability to enter into various types of chelation reactions (Harrison *et al.*, 1967).

Browning

One of the most important industrial and commercial effects of substrate oxidation by tyrosinase is the browning of plants and vegetables. The more general term polyphenol oxidase is used as several related enzymes appear to be involved.

When fruit and vegetable tissues are injured during handling, discolouration or enzymatic browning takes place. These reactions occur only in fresh living tissues or tissues that contain active enzymes. In living tissues the phenolic substrate and enzyme are separated within the cells, but come into contact on maceration (Bull and Carter, 1973). In 1931 Onslow discovered that practically all plant tissue known to darken on injury contained simple o-dihydroxyphenolic compounds. These compounds did darken faintly on exposure, but the browning was significantly faster when the plant tissue was macerated. Nelson and Dawson (1944) then proposed a theory of phenol oxidation in which the o-dihydroxyphenol was dehydrogenated, leaving o-quinones and water as end-products. The fundamental step during enzymatic browning is the oxidation of phenolic compounds into o-quinones.

Enzymatic browning is catalyzed by the polyphenol oxidase enzymes, tyrosinase and laccase, in the following way:

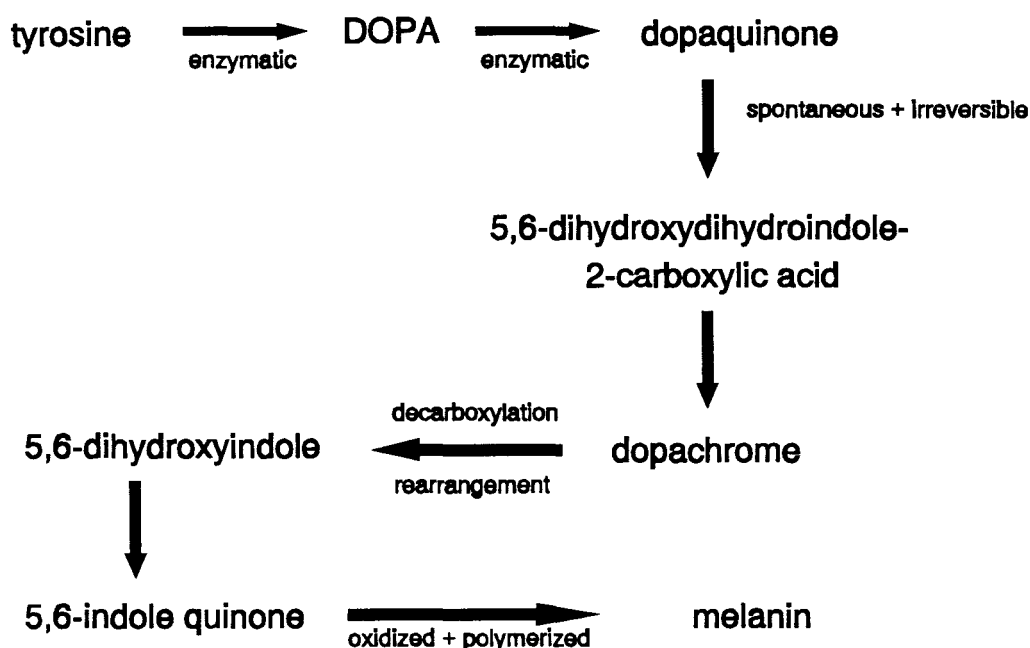


Figure 1.4: The pathway of tyrosinase oxidation in the browning reaction (Matthew and Parpia, 1971).

Products generally combine with the protein of the enzyme, thereby inactivating it. Quinones are red to reddish-brown coloured products, but the secondary reaction brings about the formation of more intensely coloured products. These secondary reactions include the following:

1. The coupled oxidation of substrates oxidized with difficulty, i.e. oxidized quinones may bring about the oxidation of other compounds which have not been oxidized directly by polyphenolases;
2. Complexing with amino compounds and proteins cause the intensification of colour during browning, e.g. the purple colour produced by tyrosinase on oxidizing catechol was identified by Jackson and Kendal in 1949 as L-proline;
3. Condensation and polymerisation. The linkage of phenols and quinones is by carbon-carbon bond formation.

It is widely known in the plant kingdom that polyphenol oxidase activity increases in response to infection or cutting. According to Farkas and Kiraly (1962) and Rubin and Artsikhovskaya (1963), the role of the enzyme in diseased tissue could be associated with the defence system of the host (cited in Hyodo and Uritani, 1967).

A study reported by Hyodo and Uritani in 1967 showed that two polyphenol oxidase enzyme components were produced in the sweet potato root tissue in response to cutting or infection by pathogens such as *Ceratosistis fimbriata*. These two components, IIIa and IIIb, were found in healthy tissue and detected on PAGE. IIIa was found in very small quantities; possibly indicating slight damage during storage. Components IIIa and IIIb are distinct from other components (IIa, IIb and IIc) as regards electrophoretic and chromatographic behaviour and enzymic properties such as pH activity curves. Actinomycin D studies showed that the enhancement in enzyme activity in cut tissue is derived from the *de novo* synthesis of enzyme protein. On infection and injury there was an accumulation of polyphenolic compounds, followed by a rapid increase in enzyme activity after a lag phase of approximately 30 hours. It was suggested that the enzyme was kept inactive in intact cells by binding with polyhydroxy-containing substances in the cellular membrane (Hyodo and Uritani, 1967).

The enzymatic browning in grapes and wine is primarily due to polyphenolase oxidation processes (Wissemann and Lee, 1980). The control of these reactions is very important in the processing of a high quality product. Enzymic oxidation during the processing of the juice influences the astringency, colour and taste of the wine. As the enzyme is found primarily in the skin of the grape, enzyme activity is highest immediately after crushing fresh grapes. Five to eight bands of enzyme activity can be seen on PAGE; some bands disappearing as the alcohol percentage increases at the end of fermentation and the enzyme is inhibited (Kidron *et al.*, 1978).

Non-enzymic forms of browning do exist. The most common cause of non-enzymatic browning involving polyphenols is the interaction between polyphenols, especially flavonoids, and heavy metals, particularly iron. The majority of these flavonoids have a β -ring hydroxylation pattern similar to catechol. Adventitious browning follows after a

mechanical injury and is fast and intense. During the normal life cycle of plants, a slow browning reaction known as functional browning takes place (Matthew and Parpia, 1971).

The prevention of enzymatic browning

The following conditions can be employed to counteract enzymic browning:

1. Heat. The enzyme is more stable in fruit than in vegetables. A temperature above 70°C is normally needed to inactivate the enzyme;
2. The most common inhibitor of enzymatic browning is sulphite in the form of SO₂ or NaHSO₃ (Scott, 1975). An exposed portion of a cut surface can be protected from browning by dipping in sulphur dioxide. Ponting and Johnson (1945) proposed that sulphur dioxide inhibits browning due to its reducing capacity i.e. it competes for oxygen and must therefore be added before the formation of quinones (cited in Scott, 1975). Embs and Markakis (1965) reported that sulphite acts as an inhibitor by combining with o-quinones and preventing their condensation to melanins;
3. Ascorbic acid acts as an anti-oxidant and reduces o-quinones before polymerization. It is not a true enzyme inhibitor, although potato enzyme undergoes marked denaturation in the presence of ascorbic acid which cannot be reversed on the addition of ionic copper (Matthew and Parpia, 1971);
4. Other methods which have been used for enzyme inhibition or inactivation include materials which form complexes with quinones e.g. meta- and tetraborates (Scott, 1975), fluorocarbon-12, and heavy metals which inhibit the enzyme by replacing copper from the enzyme (Matthew and Parpia, 1971).

Another successful method for the prevention of enzymatic browning is based on the methylation of enzyme substrates. Substrates are treated with a catechol o-methyltransferase and a methyl donor e.g. S-adenosylmethionine iodide. This blocks browning when the pH lies in the slightly alkaline range (Scott, 1975).

2-Mercaptobenzothiazole (MBT) is an exceptionally potent inhibitor of banana polyphenoloxidase. Pigment formation and oxygen uptake are simultaneously inhibited. Inhibition results from the formation of a dissociable, mixed, enzymatically inactive complex between the enzyme and inhibitor. MBT is used at very low concentrations and is therefore unlikely to produce any undesirable side effects (Palmer and Roberts, 1967).

Enzymatic browning is favourable in many industries where the production of coloured end-products and flavours associated with browning are essential e.g. tea fermentation and coffee and tobacco curing (Scott, 1975).

In tea fermentation the plucked tea leaf is repeatedly passed between rollers so that the polyphenolic substrates accumulated in the vacuoles penetrate to the enzyme without breaking the cell walls. The leaf first becomes deep red-brown, and finally darkens. Further oxidation is arrested by reducing the moisture content to 4%. In green tea these changes are prevented by an earlier inactivation of the enzyme. Oxidation appears not to proceed beyond the formation of o-quinones; this however leads to the rapid condensation of simple tannins into complex structures (Scott, 1975).

1.2. ENZYME-CATALYZED BIOTRANSFORMATIONS IN ORGANIC SOLVENTS

A number of alternative applications of the polyphenol oxidases exist, e.g. the production of fine chemicals when utilized in organic solvents. This application was investigated as an additional use for the enzyme system and required a thorough examination of enzyme activity in organic solvents.

Enzymes are very useful as reagents in chemical syntheses since they catalyze stereospecific reactions under mild conditions and transform a wide range of substrates with few side-products. Enzymes are much more reaction specific than corresponding chemical reagents. Chemical reagents may exhibit substrate specificity, but this is normally due to steric exclusion based on size, whereas enzymes discriminate more closely. Enzymes which are highly specific for one substrate, may be fairly non-specific for the cosubstrate (Cheetham, 1986).

Unfortunately stereoselectivity is affected by the reaction medium. In a non-aqueous solvent the stereoselectivity factor is reduced. The more hydrophobic the solvent, the lower the enzyme stereoselectivity (Klibanov, 1989).

The structural specificity of enzymes can be exploited to affect regiospecific reactions on molecules possessing two or more chemically similar functions. This is difficult to duplicate chemically (Cheetham, 1986).

The ability of enzymes to discriminate between enantiomers and between stereoheterotrophic groups and faces can be of value to organic chemical syntheses. The aptitude to catalyze the transformation of only one of two enantiomers is due to the difference in energies between the two possible enantiomeric transition states. The unattached isomer often binds to the active site and behaves as a competitive inhibitor (Cheetham, 1986).

The asymmetrical chemical synthesis of L-DOPA, used for the treatment of Parkinson's disease, is manufactured commercially using a chiral rhodium catalyst. Such non-enzymic reactions are very difficult to execute, give low product yields and lower optical purities than when the compound is synthesized enzymically. Due to the asymmetrical structure of enzymes they are strict chiral reagents, and the optical purity of their products is good. This is essential if regulatory authorities require the production of optically pure materials (Cheetham, 1986).

Many enzymatic processes are not feasible in water because of factors such as unfavourable thermodynamic equilibria, water-dependent side reactions and the insolubility of reactants. The catalytically active conformation of the enzyme is maintained by a delicate balance of various non-covalent interactions with water participating in all of them. The removal of water should therefore radically alter the enzyme's conformation and destroy enzymic activity. However, the enzyme cannot "see" more than a monolayer (about 1 000 molecules) of water around it. If this layer of essential water is localized and retained on the surface of the enzyme, then all the bulk water should be replaceable with organic solvents with no adverse effect to the enzyme (Klibanov, 1989). The critical water content varies from one catalyst to another and should be monitored throughout the

reaction. Polyphenol oxidase requires 0.5% volume/volume (v/v) aqueous buffer in an organic system (Khan *et al.*, 1990).

Enzyme denaturation can therefore be avoided if the reaction is carried out in a "water-water-immiscible organic solvent" biphasic system containing a non-polar water-immiscible organic solvent and/or reactants. Advantages of biphasic systems include an increased rate of reaction, improved specificity and improved kinetic parameters (Martinek *et al.*, 1981). The equilibrium is shifted in favour of the synthetic product as it is better solvated in the organic phase than the starting materials (Halling, 1987).

Monophasic organic systems are therefore those lacking a distinct aqueous phase and involve insoluble enzyme preparations in nearly anhydrous media (water content about 0.01% (Klibanov, 1989)), and water-miscible organic-aqueous cosolvents with the organic solvent as the predominant system (Dordick, 1989). Eliminating the aqueous phase facilitates the separation of the enzyme from the reaction medium and re-use of the enzyme (Boyer *et al.*, 1990). These systems can give unsatisfactory results as high solvent concentrations may lead to enzyme inhibition, decreased specificity and enzyme denaturation (Carrea, 1987).

Enzyme stability depends on the nature of the organic solvent. Solvents of low polarity and low water-solubility do not strongly affect enzyme stability (Carrea, 1987). It has been proposed that high molecular weight solvents might be less harmful to the microorganisms as they cannot penetrate the cell (Lilly *et al.*, 1987). It appears that high activity is seen in hydrophobic solvents and low activity in hydrophilic solvents. Hydrophilic solvents "strip" the essential water from the enzyme, thereby reducing enzymic activity.

The partition coefficient of an organic solvent is referred to as log P and is a measure of its hydrophobicity. In general, biocatalytic activity in organic solvents is low in relatively hydrophilic solvents having a log P < 2, moderate in solvents having a log P between 2 and 4, and high in hydrophobic solvents with a log P > 4. Therefore log P > 4 solvents do not distort the essential water layer around the biocatalyst, but leave it in an active state (Laane *et al.*, 1987).

Organic solvents can therefore affect enzymic catalysis by interacting directly with the essential water in the vicinity of the enzyme, or by interacting with diffusible substrates and products. It can cause inhibition or inactivation of the enzyme by directly interacting with the enzyme and altering protein conformation by disrupting hydrogen bonding and hydrophobic interactions. This leads to reduced enzyme activity and stability. Solvents should ideally be inert to the enzyme reaction. The damaging effects of organic solvents can be avoided by immobilizing the enzyme to retard protein-protein interactions. The stabilizing effect of immobilization improves with the number of bonds between the protein and support (Bar, 1987; Mozhaev *et al.*, 1990).

Soluble enzymes in water-miscible solvents are particularly susceptible to the effect of organic solvents. As enzymes are generally insoluble in organic solvents, they do not undergo severe inactivation (Dordick, 1989).

The compatibility of the solvent with the reaction is vital, and so also the compatibility of the reaction products with the solvent. Polar products remaining in the vicinity of the enzyme can cause product inhibition or undergo unwanted side reactions (Dordick, 1989).

The advantages of enzyme catalysis in organic systems are as follows (Antonini *et al.*, 1981; Dordick, 1989):

1. Increased solubility of nonpolar substrates.
When poorly soluble substrates are used in water, transformations have to be carried out using large reaction volumes and quantities of biological catalysts and cofactors. Product recovery also becomes difficult and impractical (Carrea, 1987);
2. Shifting of thermodynamic equilibria to favour synthesis over hydrolysis;
3. Reduction of water-dependent side reactions such as the polymerisation of quinones;
4. Immobilization may be unnecessary - enzymes are generally insoluble in organic solvents;
5. Enhanced stability of enzymes;
6. Products, if poorly soluble in water, can easily be separated from the enzyme;
7. Elimination of microbial contamination;

8. Enzymes can be solubilized in solvents by the formation of water-containing micelles, made with the use of ionic surfactants;
9. Enzymes can be used directly within the chemical process.

Attempts to use polyphenol oxidases as practical catalysts have been unsuccessful as the instability of o-quinones in water cause rapid polymerization to polyaromatic pigments. The enzyme is also inactivated by product binding. o-Quinones can be stabilized by carrying the reaction out in organic solvents and circumventing the inhibition of polyphenol oxidases by reductants. To solve o-quinone instability, quinones are continuously removed from the reaction mixture by reduction with ascorbate. The concentration of the ascorbate is kept low as it inhibits the enzyme (Doddema, 1988).

Figure 1.5 is a diagrammatic representation of the regioselective hydroxylation of phenols catalyzed by polyphenol oxidase in chloroform.

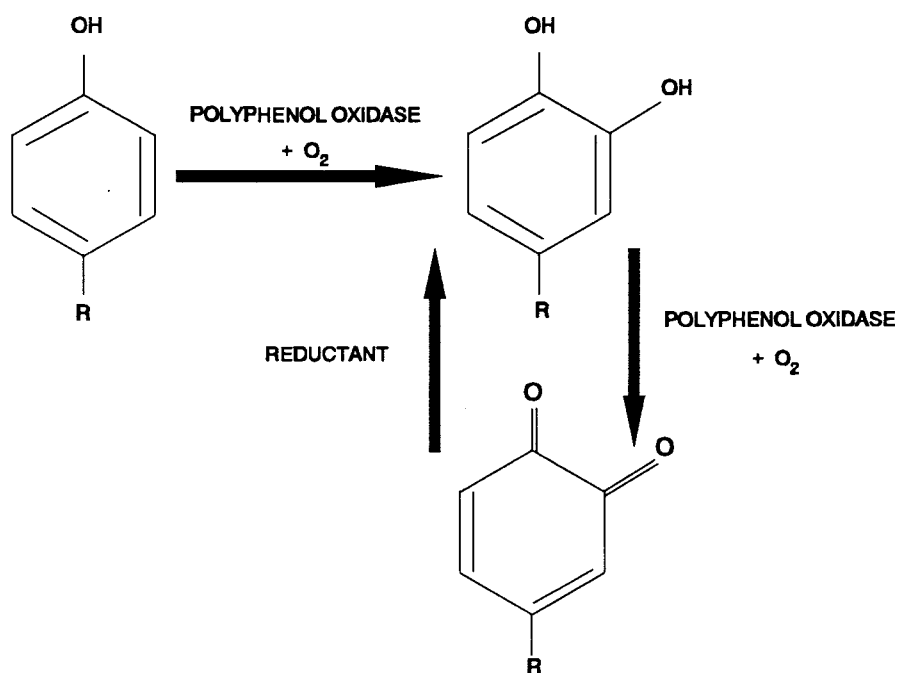


Figure 1.5: The reaction of polyphenol oxidases in organic solvents (Kazandjian and Klibanov, 1985).

Drugs produced by this system include L-DOPA from L-tyrosine. Dopamine is a transmitter for a small group of neurons associated with muscular activity in the body. A reduction in the level of dopamine in the brain leads to Parkinson's disease (Curtis, 1983). As dopamine cannot penetrate the blood-brain barrier, L-DOPA is administered and on entering the brain is converted to the neurotransmitter, dopamine (Barbeau, 1974). Victims of this disease have been shown to respond favourably when L-DOPA is administered in relatively high doses (Sih *et al.*, 1969).

D-3,4-dihydroxyphenylglycine from D-(-)-p-hydroxyphenylglycine (II) and L-epinephrine (adrenaline) from L-(-)-phenylephrine (III) can also be produced in organic media. Adrenaline is released by the adrenal medulla and is controlled by the nervous system. Its functions include increasing blood sugar levels, dilating blood vessels and increasing the rate of heartbeat (Curtis, 1983).

1.3. THE ASSIMILATION OF PHENOLIC COMPOUNDS BY MICROORGANISMS

When microorganisms grow on phenol, one or both of two processes may take place. The phenolic substrate may be degraded or dissimilated into less toxic end-products, or assimilated into cellular constituents. These two processes are catalyzed by different enzyme systems. When examining microbial growth on phenolic substrates it is essential to differentiate between these systems so as to optimize for production of selected enzymes.

A wide range of microorganisms have been recorded as being able to utilize hydrocarbons, especially paraffins, as sole carbon sources (Leonowicz and Trojanowski, 1975). The usual prerequisite for cleavage of the aromatic ring by microbial dioxygenases is that the ring contain two ortho- or para- related hydroxyl groups. Studies of the microbial oxidation of aromatic compounds show catechol to be the central intermediate and the substrate for ring cleavage by an intradiol oxygenase, pyrocatechase (catechol-1,2-oxygenase). An extradiol cleavage of catechol catalyzed by metapyrocatechase (catechol-2,3-oxygenase) was discovered in some fluorescent *Pseudomonads* and organisms isolated on cresols (Jolley *et al.*, 1969).

The most common aromatic degradations are aerobic. Organisms capable of degrading the aromatic ring include bacteria such as *Pseudomonas*, *Achromobacter*, *Bacillus* and *Arthrobacter*. Fungi include *Penicillium*, *Aspergillus* and *Fusarium* (Dart and Stretton, 1980). There have been many reports of anaerobic phenol degradation by microbial associations of fermentative/acetogenic and methanogenic bacteria (Healy and Young, 1978; Evans and Fuchs, 1988; Knoll and Winter, 1987 and 1989). Watson-Craik *et al.* (1992) reports the co-disposal of phenolic effluents and sludges with domestic, commercial and industrial refuse in landfills. When available as the sole source of carbon, catechol and phenol are completely degraded by methanogenic populations and converted to methane and carbon dioxide. The source of the hydroxyl group for these degradation reactions is water. Benzene and toluene degradation occur via a hydroxylation reaction to phenol and cresol (Evans and Fuchs, 1988).

Prokaryotes (bacteria) hydroxylate aromatic compounds using dioxygenase enzymes which catalyze a cycloaddition reaction with molecular oxygen to yield a dioxetan. This is reduced to a *cis*-diol after which oxidation to catechol takes place. In contrast, eukaryotes (fungi, yeasts and higher organisms) utilize mono-oxygenases to give a dihydroarene epoxide intermediate which is rapidly hydrolysed to a *trans*-diol. Oxidation again yields catechol (Davies *et al.*, 1989).

The following benzene derivatives are degraded by routes via catechol formation. Meta-substituted derivatives are degraded more slowly than ortho- and para- substituted compounds:

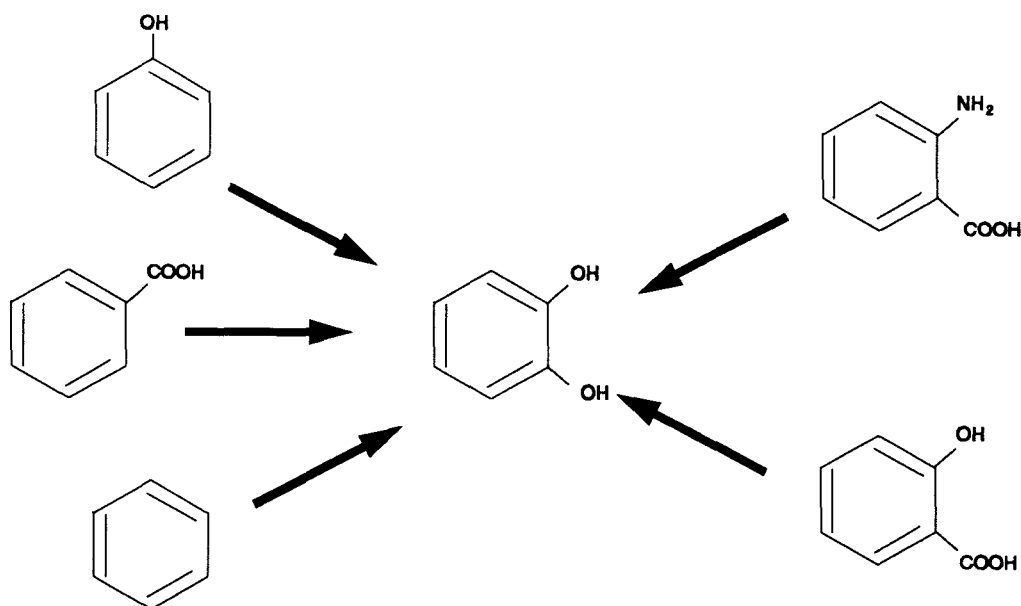


Figure 1.6: Benzene derivatives degraded via catechol (Cripps and Watkinson, 1977).

These degradative procedures introduce the two modes of oxidative cleavage of the benzene nucleus:

1. The ortho- or β -ketoadipate pathway: The bond between adjacent carbon atoms carrying hydroxyl groups is cleaved and cleavage products are metabolized by this pathway. Catechol from benzoic acid is metabolized by the catechol-1,2-dioxygenase to *cis,cis*-muconic acid. Bacteria e.g. *Pseudomonas putida*, are usually unable to metabolize ionized compounds such as *cis,cis*-muconate (Reineke, 1984). The o- pathway is only induced in the absence of an inducer of the meta- pathway (Dart and Stretton, 1980);
2. The meta- and gentisate pathways: Meta- cleavage is when the benzene nucleus is attacked between two carbon atoms, one carrying a hydroxyl group, the other unsubstituted or substituted with a group other than hydroxyl. Hydroxyls may be o- or p- related. o-Dihydroxy compounds such as catechol and protocatechuic acids are metabolized by catechol-2,3-dioxygenase. Cleavage products are metabolized

by the meta- pathway. Phenol acts as an inducer for the entire suite of meta-degrading enzymes (Dart and Stretton, 1980).

When hydroxyl groups are para- related as in gentisic acid, cleavage is catalyzed by gentisate-1,2-dioxygenase and cleavage products are metabolized via the gentisate pathway to fumaric and pyruvic acid. The alternative pathway (found in *Pseudomonas alcaligenes*) is to maleic acid and pyruvic acid. Gentisic acid may be formed as an intermediate during the degradation of compounds such as m-cresol (Bayly and Barbour, 1984).

Catechol may therefore be metabolized by either the o- or m- pathway; some bacteria have the genetic capability to degrade aromatics by both pathways. The metabolic properties of a microorganism are related to the molecular structure of its carbon source or substrate (Perez *et al.*, 1990). The precursor of catechol on which the microbe is grown therefore determines which pathway will be followed e.g. the conversion of catechol and protocatechuate to β -ketoadipate by the ortho- pathway (figure 1.7) in *P. putida* was induced by growing cells in the presence of benzoate (Orriston and Stanier, 1966).

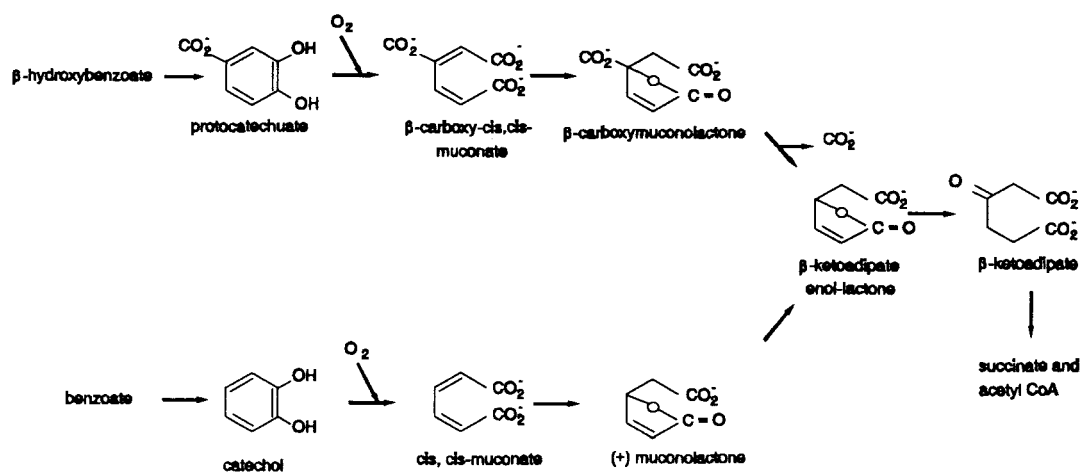


Figure 1.7: The ortho- pathway for catechol and protocatechuate degradation in *P. putida* (Orriston and Stanier, 1966).

β -ketoadipate has an intermediate role in the oxidative metabolism of aromatic compounds by bacteria. An alternative pathway for protocatechuate to β -ketoadipate has been identified in *N. crassa*. At least two steps are biochemically different in the fungus and bacteria (Orrison and Stanier, 1966).

A non-specific enzyme sequence of the meta- cleavage pathway is induced by monohydric phenols. It catalyses the oxidation of phenol and its analogues to pyruvate, a fatty acid and a carbonyl compound (figure 1.8). Phenol is therefore inducer as well as substrate (Ribbons, 1970).

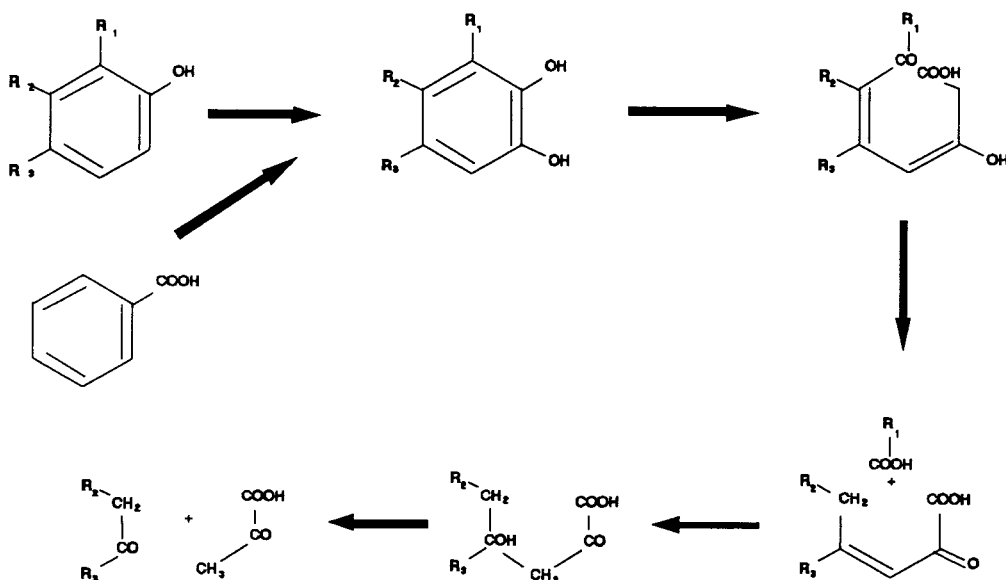


Figure 1.8: Oxidation of monohydric phenols to pyruvate by the meta- cleavage pathway (Ribbons, 1970).

The meta-cleavage pathway is a fairly non-specific means of oxidizing several phenolic analogues to common cellular metabolites such as pyruvate (Ribbons, 1970). It degrades a wider range of compounds than the ortho- pathway as the enzymes have a low specificity. This is a property of several microbial genera e.g. *Pseudomonas*, *Acinetobacter*, *Bacillus*, *Alcaligenes* and *Nocardia*. The first report of meta- pathway presence in eukaryotic microorganisms was made by Cain *et al.* in 1968 (cited in Bayly

and Barbour, 1984). They had obtained tentative evidence that protocatechuate was metabolized by a species of *Penicillium*.

The degradation of phenol to acetyl-coA and succinate by Bacterium NCIB 8250 (previously known as *Vibrio* 01) using catechol-1,2-oxygenase of the ortho- pathway, is unusual as phenols are generally metabolized by the meta- pathway (Beveridge and Tall, 1969).

The presence of an ether link, chlorine atoms, branched carbon chains or substituted amino groups in a molecule often confers increased resistance to microbial attack. More than two hydroxyl groups on the ring or the appearance of methoxyl or phenoxyl groups make a compound very resistant to oxidation (Chambers *et al.*, 1963). These groups are found in pesticidal molecules and their residues. The number, type, position, size and complexity of substituents have a marked effect on the rate of degradation of a molecule (Cripps and Watkinson, 1977).

1.3.1. The disposal of halogenated aromatic compounds

Most halogenated aromatic compounds are environmentally foreign compounds and are therefore not susceptible to biological transformation. Fortunately microorganisms have evolved an extensive range of enzymes and pathways to degrade a wide array of these compounds. These chemicals are therefore subject to non-enzymatic and enzymatic systems in an attempt to break down the organic molecule to inorganic components which can be used as carbon and energy sources (Reineke, 1984).

Some phenol-grown strains of *P. putida* act on a number of halogen-substituted catechols, but the products of ring cleavage are not metabolized further. Ring-cleavage substrates and/or products may be more lethal or inhibitory to the microorganism than the original substrate (Bayly and Barbour, 1984). Toluene-grown *P. putida* oxidizes chlorobenzene to halogenated catechols substituted in the 3-position. These compounds cannot be metabolized by the microorganism as they inactivate catechol-2,3-dioxygenase, either by chelating the iron in the active site or by active site labelling with resulting acylhalides.

Microorganisms may also lack an appropriate enzyme or set of enzymes to oxidize the compounds.

Rhodococcus chlorophenolicus is a yeast-grown polychlorophenol degrader. It degrades a number of chlorophenols with 2-4 chlorine substituents e.g. pentachlorophenol. Enzymes responsible for degradation are not constitutively expressed, but substrate induced (Apajalahti and Salkinoja-Salonen, 1986).

Minor changes to oxidizable compounds can produce recalcitrant analogues. Often enzymes can accommodate the halogen in the active site, but are unable to deal with the bulkier analogues containing other halogen atoms. Catechol-1,2-dioxygenases of the ortho-pathway are inefficient catalysts for ring cleavage of halogenated compounds due to steric and electron-withdrawing substituent effects (Reineke, 1984). Haloaromatic compounds are generally partially degraded, and not eliminated.

For the complete degradation of haloaromatic compounds the halide must be replaced by a hydroxyl group on the aromatic ring. A *Pseudomonas* sp. capable of utilizing 3-chlorobenzoate as sole carbon source has been isolated. The halide can also be replaced by an aliphatic intermediate of the haloaromatic compound. A common feature is the elimination of a halide after the o-cleavage of the halocatechol. The degradation of halogen substituted aromatic compounds cannot be expected to follow the known reaction pathways for the unsubstituted parent compounds (Reineke, 1984).

Polychlorinated biphenyls are worldwide environmental pollutants. A few microorganisms capable of metabolizing biphenyls have been isolated. *Pseudomonas* sp. NCIB 10643 and *Nocardia* sp. NCIB 10503 dissimilate biphenyl via oxidation to 2,3-dihydroxybiphenyl, followed by meta- cleavage to a product identified as 2-hydroxy-6-oxo-phenylhexa-2,4-dienoate, which is then cleaved to benzoate. In *Nocardia*, benzoate is catabolized to catechol and then to *cis,cis*-muconate. Benzoate is the final product in *Pseudomonas* oxidation. This is the first report of the catabolism of biphenyl in an actinomycete (Smith and Ratledge, 1989).

Pseudomonas sp. N31 isolated from soil uses 3-nitrophenol and succinate as sole source of nitrogen and carbon respectively. It can also use 2-nitrophenol or 4-chloro-2-nitrophenol, accumulating catechol and 4-dichloro-catechol respectively (Bruhn *et al.*, 1988).

Nitro- and chloronitroaromatic compounds are highly toxic to all organisms. In man and animals it is enzymatically reduced to carcinogenic nitroso and hydroxyl amino compounds. The latter is converted to methaemoglobin which is unable to bind oxygen (Bruhn *et al.*, 1988). Several *Pseudomonas* spp. have been isolated which aerobically reduce a variety of nitroaromatic compounds by means of a constitutive nitroreductase system (Schackmann and Müller, 1991).

1.3.2. Tyrosine phenol lyase

There is another system available to bacteria for the degradation or removal of L-tyrosine. As tyrosine is the natural substrate for tyrosinase, it is often included in culture media to "substrate induce" tyrosinase production.

Crystalline L-tyrosine phenol lyase (deaminating) was prepared from the cell extract of *Escherichia intermedia* A-21 grown in a medium supplemented with tyrosine. This enzyme catalyses the stoichiometric conversion of L-tyrosine into phenol, pyruvate and ammonia, using pyridoxal phosphate as coenzyme. It was first seen in the cell extract of *Bacterium coli phenologenes*. The tyrosine-inducible enzyme was subsequently named β -tyrosinase by Uchida *et al.* in 1953 (cited in Kumagai *et al.*, 1976). Tyrosine phenol lyase is therefore induced in tyrosine-containing media, but unlike tyrosinase, requires the presence of a coenzyme for activity (Kumagai *et al.*, 1976).

1.4. RESEARCH OBJECTIVES

Wiseman (1986) identified a number of requirements that enzyme-based processes which are to be used industrially and commercially should fulfil:

1. Enzymes should be readily available in large quantities and production costs should be minimal;
2. The enzyme process employed should be simple, robust and cheap to harness and run;
3. Enzymes must be stable. Maximum conversion of substrate to product must be attained i.e. the thermodynamic equilibria must be shifted to the formation of products. Highly active enzymes reduce reactor size and volume, reaction time and enzyme quantities required for the conversion process;
4. Product isolation and removal must be simple and practical;
5. Enzymes must be approvable for use by the regulatory authorities.

The key question in the evaluation of an enzyme for industrial use is a cost/benefit analysis. Although the use of enzymes can be expensive, costs can be offset by the ease of operation. Enzymic syntheses are generally faster than chemically-catalyzed reactions. The cost of commercial enzymes depend largely on the ease of enzyme isolation from its natural source, together with the purpose for which it is intended (Davies *et al.*, 1989).

A wide range of polyphenolase sources have been reported in the literature (Law, 1955; Bull and Carter, 1973; Held and Kutzner, 1990), but few comply with the requirements of a successful commercial system i.e. cost effectiveness coupled with high phenol removal efficiencies. The tyrosinase of the *Agaricus bisporus* fruiting body is very efficient at removing phenolic compounds from wastewaters, but the mushroom is a luxury food item and a high-cost enzyme source. It was therefore decided to screen a range of microorganisms for an alternative enzyme source that would be both efficient and economically viable.

By reviewing the long history of the polyphenolase enzyme system, the following research objectives were identified for this study:

1. Screen a range of microorganisms for the presence of tyrosinase and identify the best producers;
2. Investigate mushroom mycelia and mushroom compost as alternatives to the fruiting body as an enzyme source;
3. Investigate the Streptomycetes as alternative tyrosinase producers;
4. Optimize the enzyme production of selected sources;
5. Determine the phenol removal efficiencies of selected enzyme sources;
6. Investigate the enzymology of the tyrosinase enzyme system using *Agaricus bisporus* tyrosinase;
7. Perform enzyme reaction studies in chloroform using *Agaricus bisporus* and *Streptomyces* tyrosinase to investigate the use of the enzyme in an organic solvent system;
8. Investigate the practical application of selected whole cell cultures for phenolic effluent treatment.

CHAPTER 2

MATERIALS AND METHODS

2.1. The selection of microorganisms

All microorganisms screened for the polyphenolase enzyme system are listed in appendix 1. These were selected on the basis of literature information or the habitats they occupy. The LURGI effluent sample was obtained from a LURGI gasification plant at AECl, South Africa, and the four bacterial samples designated AECl no's 7, 26, 29 and 32, from the area surrounding the plant. Environmental samples were selected from habitats in the Hogsback forests (RSA), and were designated Hogsback cultures. As *Agaricus bisporus* is a well known source of the tyrosinase enzyme, mushroom compost and *A. bisporus* mycelia were screened as potential alternative enzyme sources. Two sources of *A. bisporus* mycelia were used i.e. a pure haploid strain obtained from the Plant Protection Unit (PPU) in Pretoria (RSA), and mushroom spawn obtained from Morning Magic mushroom farm near East London (RSA). Three tyrosinase-producing Streptomycete species were screened; two wild-type strains obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM) i.e. *Streptomyces glaucescens* (DSM no. 40716) and *Streptomyces antibioticus* (DSM no. 40234), and a third genetically-engineered strain, pIJ702 in *Streptomyces lividans*. The latter strain was provided by William Bourn from the University of Cape Town (RSA).

2.2. Growth on solid substrate (plate tests)

Once microorganism selection had taken place, cultures were streaked onto growth media plates as indicated in appendix 1. For media preparation see appendix 2.

After sufficient growth, all cultures (excluding *Streptomyces*) were streaked onto glucose-phenol minimal media agar plates (g-p plates, appendix 3) containing 0.01%, 0.1% and 1% phenol respectively. Growth and colour formation was monitored. Two different solid media were utilized to optimize growth and sporulation in *Streptomyces* i.e. Malt 3 medium

(M3) and MMT media (appendix 4). MMT is a minimal medium supplemented for maximum tyrosinase expression and melanin production. For the maintenance of the plasmid in pIJ702 cultures, a 50 mg.ml⁻¹ stock solution of thiostrepton was made up in dimethyl sulphoxide (DMSO) (Cramer *et al.*, 1982) and added to agar and liquid media to a final concentration of 5 µg.ml⁻¹. Non enzyme-producing strains i.e. *S. griseus*, *S. fradiae*, *S. cattleya* and *S. rimosus*, were used as controls.

2.3. Enzyme production in batch culture systems

All shaker-flask experiments were carried out in duplicate and results expressed as a mean value.

2.3.1. Glucose-phenol shaker-flask cultures

Cultures (excluding *Streptomyces*) were selected for inoculation into liquid media shaker-flasks (appendix 3) on the basis of brown pigment production by the colonies and/or growth on 0.1% and 1% phenol agar plates. The following organisms were selected:

Neocosmospora vasinfecta

Schizophyllum commune

Sordaria fimicola

Bacillus sp.

Neurospora crassa

The following environmental cultures were selected:

AECI cultures no's 7, 26, 29 and 32

Termitomyces umkowsaanii (I'kove) stipe fungal culture

Hogsback culture no. 1 (white fungus found on dead timber)

Hogsback culture no. 7 (fungus cultured from disintegrated timber)

Suillus bovinus mycelial culture

Agaricus bisporus spawn mycelia was also inoculated into shaker-flask liquid media in an attempt to induce enzyme production.

Glucose-phenol medium containing 0.05% phenol/tyrosine was prepared (appendix 3) and 200ml aliquots dispensed into 500ml Erlenmeyer flasks. These were inoculated with 20ml overnight broth cultures of the relevant organisms (10% inoculum) and vigorously shaken at 25° or 37°C (fungi and bacteria respectively) for 5-7 days.

2.3.2. *Streptomyces* shaker-flask cultures

The following liquid media were used to induce and optimize tyrosinase production in *Streptomyces*: M3 medium, MMT medium, Yeast extract - Malt extract medium (YEME) and the complex liquid medium, GYM (glucose-yeast medium) - appendix 4.

2.4. Enzyme induction studies

2.4.1. Glucose-phenol shaker-flask cultures

A number of inducers were tested for their ability to initiate enzyme production.

2.4.1.1 Substrate induction

Substrate induction (SI) involved the use of phenol and/or tyrosine included in the growth media as described in section 2.3.1. These cultures also functioned as controls for the induction experiments listed below.

2.4.1.2. Enzyme induction by ethylene

The following cultures were selected for this experiment:

Neocosmospora vasinfecta

Schizophyllum commune

Sordaria fimicola

Bacillus sp.

Hogsback culture no. 1

Shaker-flask cultures were sparged with ethylene gas for 15 seconds, stoppered and shaken as before.

2.4.1.3. Enzyme induction by the inclusion of metabolic inhibitors

The inclusion of metabolic inhibitors in growth media was tested for inductive effects. The following cultures were selected for this experiment:

Neocosmospora vasinfecta

Schizophyllum commune

Hogsback culture no. 1

The following metabolic inhibitors were included at the indicated concentrations according to Lyr and Luthardt, 1965.

Oxytetracycline	$1 \times 10^{-4}M$
Iodoacetic acid	$1 \times 10^{-4}M$
Sodium arsenate	$3 \times 10^{-4}M$

2.4.2. *Streptomyces* shaker-flask cultures

2.4.2.1. Enzyme production in M3 medium

200ml M3 medium was dispensed into 500ml Erlenmeyer flasks and inoculated with *S. antibioticus*, *S. glaucescens* and *S. lividans* (pIJ702) cultures respectively. To determine the effect of culture inoculum on enzyme production, media was inoculated with either overnight broth cultures (10% inoculum) or spores. For the latter inoculum, sporulating cultures were harvested as follows: Plates were flooded with 20mls 20% sterile glycerol and the surface scraped with a sterile slide to release the spores. Flasks were inoculated with 100 μ l spore suspension.

Flasks were incubated with shaking at 30°C for 5-7 days. Due to heavy growth after five days, broth-inoculated cultures were passaged into a second set of shaker flasks (10% inoculum) for a further week. Biomass production and media colour in all flasks was monitored. Intracellular enzyme was extracted by ultrasonication (section 2.8.1) and assayed against tyrosine and phenol (section 2.11.1).

2.4.2.2. Enzyme production in GYM medium - Experiment a

The ability of GYM medium (appendix 4) to induce maximum tyrosinase production in *S. antibioticus* and *S. glaucescens* was assessed after the method of Gardner and Cadman (1990). This system uses a two-temperature strategy to maximise enzyme production. The culture was held at 30°C for 10 hours to build up cell biomass and then reduced to 25°C (after the lag phase) for 25 hours to induce enzyme production. After the first 10 hour period, additional carbon (in the form of 5 g.l⁻¹ glucose) and nitrogen (in the form of 0.5 g.l⁻¹ asparagine) sources were added, also methionine (10⁻³M) as enzyme inducer (Katz and Betancourt, 1988). Two media pH values, pH 7.2 and pH 9, were compared.

100ml medium was dispensed into 250ml flasks (septums fitted to base of flask to aid quick and efficient sampling) and inoculated with 10% spore suspensions (see section 2.4.2.1. for preparation) in sterile saline - 0.05% Tween 80. As tyrosinase is membrane-bound, Tween 80 was included to aid enzyme solubilization (Neugebauer, 1990). Autoclaved stainless steel coils were inserted into flasks to prevent the formation of mycelial pellets and to improve aeration. Inoculated flasks were shaken vigorously at 110 rpm. 10ml samples were taken at t=0, 10 hours, 15 hours, 20 hours, 25 hours, 30 hours and 35 hours and centrifuged as in section 2.8. Pellets were disrupted by ultrasonication to release intracellular enzyme (section 2.8.1). Intracellular and extracellular extracts were assayed for tyrosinase using L-DOPA as substrate (section 2.11.4). Glucose determinations were carried out on supernatants by the Somogyi-Nelson method (appendix 5). Biomass determinations were carried out by dry weight analysis. Whatman GF/A discs, diameter 2.5cm, were dried in a 60°C oven for 1.5 hours and weighed. Pelleted mycelia were resuspended in 10ml sterile distilled water, passed through the filters and washed twice with 10ml sterile water. Filters were then dried at 60°C for 1.5 hours, removed and reweighed.

A number of controls were used:

- Control 1: Temperature maintained at 30°C to evaluate the use of a two-temperature regime.
- Control 2: No aeration.
- Control 3: No additional carbon (glucose) or nitrogen source (asparagine) added.
- Control 4: No inducer (methionine) added.

Experiment b

This study was carried out at pH 7.2 as for experiment a, but the 30°C incubation period was lengthened from 10 to 20 hours. Samples were taken at $t = 0$, 20 hours, 28 hours, 36 hours, 44 hours and 52 hours.

2.4.2.3. Enzyme production in GYM medium using *S. lividans* (pIJ702)

150ml pH 7.2 GYM medium was dispensed into 1 litre flasks and inoculated with 5ml spores (no thiostrepton added). The spore suspension was prepared as in section 2.4.2.2. Culture time was lengthened to 72 hours. A number of optimization experiments were carried out:

- Experiment 1: A two-temperature strategy was maintained as in section 2.4.2.2.
- Experiment 2: The 30°C biomass production period was increased from 10 to 20 hours.
- Experiment 3: Culture temperature was maintained at 30°C.
- Experiment 4: Culture temperature was maintained at 25°C.

For experiments 2-4, additional glucose, asparagine and methionine were added after 20 hours. Samples were taken every 8 hours and treated as in section 2.4.2.2. Cell pellets were disrupted by ultrasonication (section 2.8.1). Enzyme assays were carried out using L-DOPA as substrate (section 2.11.4).

2.4.2.4. Enzyme production by *S. lividans* (pIJ702) in various media

This experiment was initiated to determine the media most suitable for maximal biomass and enzyme production of the plasmid-carrying strain *S. lividans* (pIJ702). 200ml pH 8 M3, YEME and MMT media (appendix 4) were each inoculated with a quarter plate of spores and shaken vigorously for 7 days at 30°C. No inducer is required in the medium as the *mel* gene carried on each copy of the plasmid is constitutively expressed (Gardner and Cadman, 1990). Media colour changes, indicating the production of melanin, were noted. After centrifugation, pellets were disrupted using a Yeda-press (section 2.8.2). Enzyme assays were carried out using L-DOPA as substrate (section 2.11.4).

2.4.2.5. The effect of pH on *S. lividans* (pIJ702) in MMT

An experiment was carried out to assess the affect of pH on the growth and enzyme production of *S. lividans* (pIJ702) in MMT medium. MMT medium was prepared and 200ml aliquots dispensed into 500ml flasks, inoculated with loopfuls of sporulating *S. lividans* (pIJ702) culture and shaken at 30°C for 5 days. Colour changes and biomass production were monitored.

The following four pH values were tested:

pH 7.2 (Kirby, pers. comm.), pH 7.4, pH 7.6 and pH 8.0 (Bourn, pers. comm.).

Intracellular enzyme was recovered using a Yeda-press (section 2.8.2). Extracts were assayed using L-DOPA as substrate (section 2.11.4). Preliminary purification by ammonium sulphate precipitation was carried out on all samples (method described in appendix 8) and enzyme assays performed after each step.

2.4.2.6. Enzyme production in pH 7.6 GYM and MMT media

This experiment compared the growth of *S. antibioticus*, *S. glaucescens* and *S. lividans* (pIJ702) in MMT and GYM media at pH 7.6. 200ml medium was dispensed into 500ml flasks, inoculated with loopfuls of spores and incubated shaking at 30°C for 5-6 days in MMT media and 80 hours in GYM media. Thiostrepton was added to cultures of *S. lividans* (pIJ702). Media colour changes and pH were monitored throughout the

experiment. 15ml samples were taken daily from MMT flasks and treated as in section 2.4.2.2. Pellets were disrupted using a Yeda-press (section 2.8.2). Extracts were assayed against L-DOPA (section 2.11.4).

Additional glucose, asparagine and methionine were added to GYM flasks after 20 hours; the temperature was then changed to 25°C for the duration of the experiment. Samples were taken every 12 hours and analyzed as in section 2.4.2.2. Pellets were disrupted using a Yeda-press (section 2.8.2). Extracts were assayed against L-DOPA (section 2.11.4).

2.5. Enzyme production over time

Initial enzyme assays suggested *Neocosmospora vasinfecta* as a potentially rich source of tyrosinase and it was therefore selected to determine the narrow "harvest windows" during which maximum enzyme production occurs.

10 glucose-phenol shaker-flasks, each containing 0.05% phenol/tyrosine, were inoculated with 10% broth cultures of *Neocosmospora vasinfecta*. These were incubated shaking at 25°C for 1-10 days. A flask was removed everyday and the enzyme extracted (section 2.8.1). Enzyme extracts were assayed against L-tyrosine (section 2.11.1). The production of enzyme over time was graphically represented.

2.6. Deep liquid culture of *Agaricus bisporus* mycelia

Mycelial cultures were maintained on malt extract agar (MEA) and subcultured into potato dextrose and Sabouraud broth media (appendix 2) once sufficient growth had been established. As mycelial growth was slow, broths were supplemented with 10% horse manure extract. This extract was prepared by collecting horse manure, adding water and simmering over a low flame for 20-30 minutes. Once cooled, the solution was filtered and the liquid used to supplement the media. Mycelial biomass production increased significantly. Cultures were incubated with shaking at 25°C for seven days after which a 10% inoculum was passaged into new media. As sterility and strain purity were essential to this study, all manipulations were carried out under strict aseptic conditions. At each passage samples were streaked onto MEA and nutrient agar (NA) to check for

contamination. Contaminated flasks were discarded. Samples were also stained with lactophenol blue to check for the development of clamp connections. Intracellular enzyme was extracted by ultrasonication (section 2.8.1) and all extracts assayed against L-tyrosine (section 2.11.1).

2.7. Mushroom compost as a source of tyrosinase

Two 5kg batches of mushroom compost were obtained from a mushroom farm - one batch collected after inoculation with spawn and the other collected after harvest. The following four enzyme extraction systems were tested: 50mM pH 6.5 phosphate buffer of potassium phosphate salts, the same buffer + ultrasonication, chloroform, and distilled water. An autoclaved compost sample was used as a tyrosinase-deficient control.

To prepare a sample for extraction it was ground with some solvent using a mortar and pestle to release any attached or intracellular enzyme. After grinding, the remaining solvent was added and the sample shaken for 1 hour. When testing ultrasonication as a method of extraction, the sample was ultrasonicated on ice before shaking. 200ml solvent was added to each 50g sample. After extraction, compost samples were centrifuged at 10 000g and 4°C for 30 minutes in a SORVALL RC-5 Superspeed Refrigerated Centrifuge using a GSA rotor. The supernatant was collected and stored at -20°C for assay against tyrosine (section 2.11.1).

2.8. Enzyme extraction from batch culture systems

On the completion of enzyme production in liquid media, culture media or media samples were collected and centrifuged in refrigerated centrifuges - either a SORVALL RC-5 Superspeed Refrigerated Centrifuge using a GSA rotor for culture media and SS-34 rotor for samples, or a Beckman J2-21 Refrigerated Centrifuge using a JA-14 rotor for culture media and JA-20 rotor for smaller samples. Samples were centrifuged at 4°C for 20 minutes at 10 000-12 000 rpm and supernatants collected as the extracellular extract. The pellet contained the intracellular fraction and was disrupted either by ultrasonication or a Yeda-press.

2.8.1. Ultrasonication

All glucose-phenol shaker-flask culture pellets were resuspended in 5-10ml pH 6.5 50mM potassium phosphate buffer and disrupted using a MSE ultrasonicator set at maximum frequency. *Streptomyces* pellets were resuspended in the same buffer if assayed against tyrosine or in pH 6.0 0.1M sodium dihydrogen phosphate if assayed against L-DOPA. Samples were kept on ice throughout the extraction process.

2.8.2. Yeda-press

Most of the *Streptomyces* cell samples were fractured by a Yeda-Press using compressed nitrogen applied to the sample in the cell.

Pellets were resuspended in cold pH 6.0 0.1M sodium dihydrogen phosphate (in preparation for L-DOPA assay), kept on ice throughout extraction with the pressure cell refrigerated before use. Five passages at a pressure of 2 000 Psi were sufficient to rupture cells and release the enzyme. After disruption samples were recentrifuged to pellet broken cells, leaving the intracellular enzyme extract in the supernatant.

2.9. Crude tyrosinase extraction from *Agaricus bisporus*

A crude enzyme extract was prepared from the common field mushroom *Agaricus bisporus*, as described by Atlow *et al.* (1984).

340g mushrooms were cut up and soaked in 1.25 litres pre-cooled acetone. The suspension was homogenized in a blender and the pulp separated by filtration through Whatman folded filter papers. The resultant paste was squeezed between two layers of Whatman 3MM Chr filter paper until dry, and frozen using liquid nitrogen. The frozen mass was then broken into small pieces, suspended in 150ml distilled water and incubated at 4°C overnight. The suspension was filtered through cheesecloth and the filtrate collected as the enzyme extract.

2.10. Spot tests

L-tyrosine and p-cresol can be used as test substrates for detecting the presence of tyrosinase (Marr *et al.*, 1986). 1% (v/v) p-cresol was prepared, warmed to dissolve the substrate and a drop added to 200 μ l aqueous sample at room temperature. Ethanol (95%) and distilled water were used as control reagents. The formation of a brownish-red colour (detectably different from the controls) within 15 minutes indicated a positive result. p-Cresol was chosen as substrate as it gave a faster and more intense colour reaction than L-tyrosine.

2.11. Enzyme assays

Enzyme assays were carried out in triplicate and results expressed as a mean value. A number of assay methods were employed for tyrosinase. One of three spectrophotometers - either a Bausch and Lomb Spectronic 1001, a Pye Unicam SP8-400 UV/VIS or a Spectronic 20 - was used. Controls included the elimination of either enzyme extract or substrate from the assay mixture.

2.11.1. L-tyrosine as assay substrate

This method was adapted from Atlow *et al.* (1984) and enzyme activity calculated from the following specifications: One unit of tyrosinase will cause an increase in absorbance at 280nm of 0.001 per minute while maintained at pH 6.5 and 25°C in a 3ml reaction mixture containing L-tyrosine (SIGMA catalogue - tyrosinase: EC 1.14.18.1).

To maintain the pH of the reaction mixture, 0.5mM L-tyrosine was prepared in a 50mM pH 6.5 phosphate buffer of potassium phosphate salts. The reaction mixture was adjusted to a total volume of 4ml i.e. 0.13ml enzyme extract and 3.87ml tyrosine substrate, aerated and absorbances read every 30s using a Pye Unicam SP8-400 UV/VIS spectrophotometer set at 280nm. Spectrophotometer settings were as follows:

Band width	1nm
Wavelength exp (nm/cm)	10

Recorder range	2.0A
Wavelength	280nm

This method was also adjusted to assay for the enzyme against 0.5mM phenol and a mixture of phenolics i.e. 0.5mM tyrosine and phenol (1:1). Results were expressed as units of activity.ml⁻¹ extract and units of activity.mg⁻¹ protein.

2.11.2. Catechol as assay substrate

This assay method, adapted from Wissemann and Lee (1980), uses catechol as assay substrate.

20ml pH 6.5 0.01M sodium phosphate buffer was prepared and pipetted into a 50ml flask. 2ml 0.5M catechol was added and the reaction mixture left incubating at room temperature for 30 minutes. 1ml enzyme extract was added, mixed and a 4ml aliquot transferred to a cuvette. Absorbances were read every 30s at 420nm on a Pye Unicam spectrophotometer.

2.11.3. p-cresol as assay substrate

This enzyme assay uses p-cresol as substrate and was adapted from Kazandjian and Klibanov (1985).

0.5mM p-cresol was prepared in a pH 6.5 50mM phosphate buffer of potassium phosphate salts. The assay was carried out as for section 2.11.1, the wavelength being adjusted to 395nm to detect for the formation of 4-methyl-1,2-benzoquinone.

2.11.4. L-DOPA as assay substrate

This assay method, described by Gardner and Cadman (1990), uses L-β-3,4-dihydroxyphenylalanine (L-DOPA) as enzyme substrate and was used primarily for the assay of *Streptomyces* tyrosinase.

10mM L-DOPA was prepared in 3ml pH 6 0.1M sodium dihydrogen phosphate. 100 μ l enzyme extract was added to the substrate which was maintained at 30°C and an increase in absorbance at 475nm was monitored over 10-30 sec. intervals.

2.12. The determination of protein concentration

2.12.1. The Folin-Lowry method

This method of protein determination is based on the formation of a copper-protein complex in an alkaline environment. Folin-Ciocalteu reagent, a phosphomolybdic-phosphotungstate reagent, is then reduced by this complex to yield an intense blue colour which is determined spectrophotometrically (Lowry *et al.*, 1951). The method is described in appendix 6.

2.12.2. Bradford's method

This method is based upon the interaction of protein and Bradford's Protein Assay Reagent (Bradford, 1976). Bradford's Reagent contains 0.01% Coomassie Brilliant Blue G 250, 4.7% ethanol and 8.5% phosphoric acid (appendix 6).

2.13. Zymograms as a detection system for tyrosinase

A quick and efficient system is needed for detecting polyphenolase enzymes. Spot tests and enzyme assays are of limited sensitivity and zymogram analysis presents a potential alternative.

Protein determinations and enzyme assays were carried out on all samples before loading gels. When possible, approximately 30 μ g protein was loaded.

7.5% and 3.0% polyacrylamide gels were prepared as described by Laemmli (1970). 7.5% gels were set up on a vertical (Hoefer, model SE 280) "Tall Mighty Small" mini-gel system (appendix 7). Aqueous and/or dried crude *A. bisporus* tyrosinase extract was run as a positive control; samples were loaded using a 100 μ l Hamilton syringe. Dissociation buffer

was loaded on to gels so as to determine the position of the solvent front. 15% glycerol was added to all samples to increase density and prevent leakage out of wells and into bath buffer. Samples were untreated so as to retain enzyme activity. Gels were run in a 4°C room at 150V for approximately 2.5 hours. After electrophoresis, gels were placed in aqueous or organic substrates (appendix 7) for 30 minutes to 1.5 hours to develop enzyme 'spots' i.e. brown melanin formation due to enzyme-substrate interaction. After enzyme development, gels were placed in 10% trichloroacetic acid (TCA) for 20 minutes to fix the protein, rinsed in tap water and dried on a gel drier. Once dry, gels were photographed. Gels run from top to bottom on photographs.

2.14. Preliminary enzyme purification using salt fractionation

Pilot-scale fractionation experiments were carried out to determine the percentage of salt required to precipitate maximum enzyme and minimum extraneous protein. Percent of saturation with ammonium sulphate ranged from 40% to 70%. After each salt addition protein concentration and enzyme activity was determined (see appendix 8 for method).

The partial purification of enzyme extracts by salt fractionation was necessary to prevent enzyme inhibition by enzyme-product binding (appendix 8). Enzyme activity was determined after each step. All stages were carried out at 0-4°C to minimize protein denaturation.

Salt fractionation is based on the fact that the solubility of most proteins decrease at high electrolyte concentrations. Multivalent ions such as sulphate are more effective at concentrating proteins than monovalent ions like chlorine. Ammonium sulphate is most commonly used as it is highly water soluble, can be obtained in a high degree of purity, is cheap and has no detrimental effect on the structure of proteins (Williams and Wilson, 1981).

2.15. Characteristics of *Agaricus bisporus* crude tyrosinase

2.15.1. Enzyme stability

The extracted enzyme appeared to lose activity if stored at 4°C for periods longer than a few days. Storage at -20°C therefore had to be considered, and enzyme stability after continual freezing and thawing had to be determined. Enzyme activity was tested using L-tyrosine as assay substrate (section 2.11.1).

2.15.2. The influence of temperature

The activity of crude tyrosinase was tested over a range of temperatures up to 37°C. Assay method 2.11.1 was used and the temperature of the reaction mixture adjusted accordingly.

2.15.3. The influence of pH

Enzyme activity was tested over the range pH 6-8. Assay method 2.11.1 was used. 50mM phosphate buffer of potassium phosphate salts was prepared and the pH adjusted as required.

2.15.4. Enzyme substrate specificity

The following substrates, excluding L-DOPA, were tested using assay method 2.11.1 to determine the specificity of mushroom tyrosinase: DL-tyrosine, L-tyrosine, phenol, catechol, p-cresol, L-DOPA. L-DOPA was tested using assay method 2.11.4.

2.16. Enzyme inhibition studies

To test Scott's (1975) theory of "reaction inactivation" i.e. the oxidation of substrate stops long before all the substrate has been utilized completely, enzyme-substrate exhaustion studies were carried out using *Neocosmospora vasinfecta* extracellular enzyme extract.

Firstly 'double enzyme' reactions were carried out. 0.227ml enzyme extract, i.e. double the normal volume, was added to 3.386ml tyrosine substrate prepared as in section 2.11.1, and the reaction monitored at 280nm.

'Double substrate' reactions were also carried out. 1mM, versus the normal 0.5mM tyrosine, was prepared in 50mM pH 6.5 phosphate buffer of potassium phosphate salts and 0.113ml enzyme extract added. The reaction was followed at 280nm and results represented graphically.

2.17. Kinetics of *Agaricus bisporus* crude tyrosinase

Lineweaver-Burke and Michaelis-Menten plots were constructed to determine K_m and V_{max} values for *Agaricus bisporus* tyrosinase on the following substrates: L-tyrosine, p-cresol and L-DOPA. 0.0625mM to 0.5mM L-tyrosine and p-cresol, and 0.1mM to 10mM L-DOPA substrates were prepared and assays carried out as in sections 2.11.1, 2.11.3 and 2.11.4 respectively.

2.18. Phenol removal efficiencies

This method was adapted from Atlow *et al.* (1984). Once tyrosinase-containing samples had been identified, it was essential to determine their phenol removal efficiencies under experimental conditions. 50 mg.l⁻¹ phenol was prepared in 200ml 50mM pH 8.0 phosphate buffer of potassium phosphate salts. As this is a highly oxidative reaction, constant agitation and aeration was provided by stirring continuously on a magnetic stirrer and bubbling air through the medium using a small filter pump. Small air bubbles ensured maximum oxygen transfer to the medium. 500ml Erlenmeyer flasks were adjusted to provide a sampling port, inlet for air and an outlet to prevent pressure build-up once the flasks had been stoppered - plate 2.1.

1ml samples were taken at the following times throughout the 5 hour experiment: t=0 (i.e. enzyme addition), 15 sec., 30 sec., 1 minute, 1.5 minutes, 2 minutes, 5 minutes, 10 minutes, 15 minutes, 30 minutes, 1.5 hours and 5 hours. Most of the samples were taken at the beginning of the experiment as the reaction takes place quickly in aqueous

media. Once removed, samples were quickly placed in a 90°C water bath to stop the reaction and inactivate the enzyme. Samples were either analyzed immediately or stored at -20°C for later analysis. Colour changes were carefully monitored throughout the reaction.

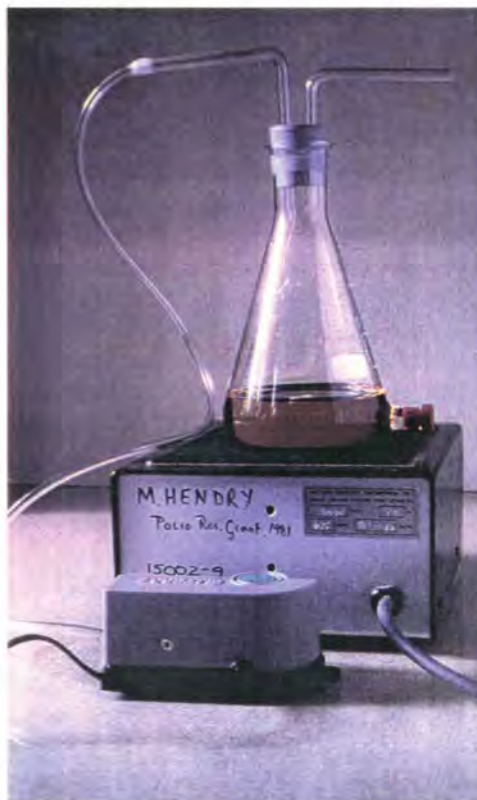


Plate 2.1: Apparatus used for aeration experiments.

The following enzyme extracts were chosen for phenol removal studies: (Enzyme units added are approximated amounts. Activity was determined by assaying against L-tyrosine - section 2.11.1.).

1. *Agaricus bisporus* crude enzyme extract - 161 000 units of activity added to phenolic substrate. Immediate sample analysis.
2. *Agaricus bisporus* crude enzyme extract - 161 000 units of activity added. Samples stored at -20°C for later analysis.
3. *Agaricus bisporus* crude enzyme extract, reaction media pH 8.0 - 14 500 units of activity added.
4. *Agaricus bisporus* crude enzyme extract, reaction media pH 6.0 - 14 500 units of activity added.

5. Commercial tyrosinase - 14 500 units of activity added.
6. Hogsback culture no. 1, extracellular extract grown with the metabolic inhibitor oxytetracycline - 14 000 units of activity added.
7. AECI culture no. 32, extracellular extract - 18 000 units of activity added.
8. AECI culture no. 26, intracellular extract - 16 000 units of activity added.
9. *Agaricus bisporus* mycelia cultured in Sabouraud broth - A7, extracellular extract - 14 500 units of activity added.
10. Mushroom spawn mycelia cultured in Sabouraud broth - C1, intracellular extract - 14 500 units of activity added.
11. Mushroom compost (collected after harvest), enzyme extracted using ultrasonication in pH 6.5 phosphate buffer - 16 000 units of activity added.
12. Mushroom compost (collected after harvest), enzyme extracted with chloroform - 14 000 units of activity added.

For experiments 11 and 12, samples were taken at time 0, 5 minutes, 15 minutes, 30 minutes, 4 hours and 5 hours.

Enzyme/buffer or distilled water/phenolic substrate phenol removal systems were set up as controls.

2.19. Enzyme catalysis in organic media

These experiments were carried out as for aqueous phenol removal experiments, the phosphate buffer being replaced with HPLC grade chloroform. Un-aerated systems were also tested as chloroform has a higher oxygen carrying capacity than water and additional aeration was unnecessary. The un-aerated, closed system prevented the loss of organic substrate by evaporation.

Samples were taken at the following times as the enzyme reaction is much slower in organic solvents: $t=0$ (i.e. enzyme addition), 15 minutes, 30 minutes, 1 hour, 1.5 hours, 2 hours, 2.5 hours, 3 hours, 3.5 hours, 4 hours, 4.5 hours, 5 hours, 5.5 hours and 6 hours.

The following enzyme extracts were selected for the experiments in chloroform:

(Enzyme units added are approximate values. Activity was determined by assays against L-tyrosine - section 2.11.1)

1. *Agaricus bisporus* crude enzyme extract - aerated system - 14 500 units of activity added. Sampling times as in section 2.19.
2. *Agaricus bisporus* crude enzyme extract - unaerated (closed) system - 14 500 units of activity added.
3. Mushroom compost (collected after harvest), enzyme extracted with chloroform - 14 000 units of activity added.
4. *Agaricus bisporus* crude concentrated enzyme extract - 2 755 units of activity added. Modified aeration experiment. Samples were concentrated by freeze-drying.
5. *Streptomyces antibioticus* broth 2 culture, intracellular extract - 2 755 units of activity added. Modified aeration experiment.
6. *Streptomyces antibioticus* broth 2 culture, intracellular concentrated extract - 2 755 units of activity added. Modified aeration experiment.
7. *Streptomyces lividans* (pIJ702) broth 2 culture, intracellular concentrated extract - 2 755 units of activity added. Modified aeration experiment.

Modified aeration experiments were carried out as follows:

The dried enzyme sample was dissolved in a drop of pH 7 50mM phosphate buffer of potassium phosphate salts and placed into a 20ml round-bottomed flask. 2ml 25mM phenol dissolved in chloroform was added, the flask stoppered and stirred vigorously. 50 μ l samples were removed at hourly intervals for 4-5 hours. A granule of anhydrous Mg₂SO₄ was added to samples to absorb water before analysis on capillary-GC (SE 30 column) was carried out.

2.20. Product analysis

Samples collected during phenol removal and experiments in chloroform had to be analyzed and products identified. Different analytical separation techniques available for aromatic compounds were therefore investigated. The following systems were investigated and methods optimized.

2.20.1. High pressure liquid chromatography (HPLC)

High pressure liquid chromatography (HPLC) has been used by a number of researchers for the separation and identification of tyrosinase products during the oxidation of various substrates (Olano and Hernandez, 1985; Jacobsohn *et al.*, 1988).

To achieve maximum separation of compounds, the following acetonitrile:water mobile phase conditions were used. Samples were analyzed using a Pye Unicam liquid chromatogram with a model PU4020 detector and a 25 x 0.4cm reverse-phase μ Bondapak- C_{18} column.

1. 5% acetonitrile in water
2. 40% acetonitrile in water
3. 60% acetonitrile in water
4. 70% acetonitrile in water
5. 80% acetonitrile in water
6. 5-40% acetonitrile in water
7. 40-80% acetonitrile in water
8. 5-80% acetonitrile in water

Mobile phases 1-5 were run isocratically, whereas 6-8 were gradients. Phenol, p-cresol, p-quinone (o-quinone was unavailable) and catechol were used as standards at concentrations ranging from 200 - 1 000ppm. Standards were also run at two different wavelengths, 254nm and 275nm, in an attempt to optimize the system for the separation of phenolic compounds.

2.20.2. Gas-liquid chromatography (GC) - packed columns

The advantages of GC over HPLC are sensitivity and speed (Williams and Wilson, 1981). Once a column has been conditioned, samples can be sequentially injected and efficiently analyzed. Two columns were considered for their ability to separate aromatic compounds, i.e. a 80/100 Carbowack C/O, 1%/SP-1000 column and a Tenax column. The Carbowack

system separates o-, m- and p-cresol isomers, but is unable to identify other intermediates and was therefore unsuitable for the analysis of mixed phenolic samples.

Phenol, p-cresol, p-quinone and catechol standards were successfully separated on a Hewlett Packard 5830A gas chromatograph with a flame-ionization detector (FID) using a Tenax column.

2.20.2.1. Tenax

Tenax is a porous polymer column packing material that is based on 2,6-diphenyl-p-phenylene oxide (Alltech Associates Handbook, 1982). This system was found to be successful for the analysis and separation of phenolic compounds. As no derivatisation was required, samples could be injected directly onto the column. The following chromatographic conditions were used.

Temperature 1	210°C
Oven temperature	210°C
Injection temperature	250°C
FID temperature	250°C
Oven max.	260°C
Time 1	10.0 minutes
Chart speed (cht spd)	1.0 cm.min ⁻¹
Attenuation (attn) 2↑	4
Slope sensitivity (slp sens)	0.6
Area reject (rej)	500
Flow	33.0 ml.min ⁻¹

All systems used nitrogen as the mobile phase.

2.20.3. Gas-liquid chromatography (GC) - capillary columns

A number of capillary columns were evaluated for their ability to separate phenolic standards and identify reaction intermediates. A Hewlett-Packard 5790 Gas Chromatograph with a FID was used.

2.20.3.1. Carbowax 20M capillary column

Aqueous samples could not be injected onto this column. Samples therefore had to be dried and resuspended in an organic solvent. The following chromatographic conditions were used.

Temperature 1	-a range of isothermal runs were done from 120°-190°C
Oven temperature	-as above
Oven max.	225°C
Injection temperature	200°C
FID temperature	200°C
Final time	30.0 minutes
Flow	15.0 ml.min ⁻¹

Only the catechol standard was detected.

2.20.3.2. OV-225 capillary column

The following chromatographic conditions were used.

Initial temperature	100°C
Final temperature	180°C
Rate	10°.min ⁻¹
Oven temperature	100°C
Injection temperature	200°C
FID temperature	200°C

Oven max.	220°C
Final time	40.0 minutes
Flow	15.0 ml.min ⁻¹

Only quinone and catechol standards were detected.

2.20.3.3. SE 30 4% 5 μ capillary column

Aqueous samples could not be injected onto this column. Samples were therefore dried under nitrogen to prevent oxidation and reconstituted in chloroform before injection. The following chromatographic conditions were used.

Initial temperature	140°C
Oven temperature	140°C
Final temperature	180°C
Rate	7°.min ⁻¹
Injection temperature	220°C
FID temperature	220°C
Oven max.	300°C
Initial time	6 minutes
Final time	10-15 minutes
Flow	30.0 ml.min ⁻¹

The integrator was set as follows:

attn 2↑	0
threshold (thrsh)	0
area rej.	0
peak width (pk wd)	0.16
cht spd	0.5 cm.min ⁻¹

All standards were prepared in 99.9% pure chloroform at the following concentrations:

phenol and p-quinone	0.05 mg.ml ⁻¹
catechol and p-cresol	0.5 mg.ml ⁻¹

Standards were detected at the following retention times:

quinone	5.10 minutes
phenol	6.31 minutes
p-cresol	7.38 minutes
catechol	12.02 minutes

All systems used nitrogen as the mobile phase.

2.20.4. Ultra-violet spectroscopy

In addition to sample analysis by GC, standards and samples were scanned (190-322nm) by ultra-violet spectroscopy (UV) on a Pye Unicam SP8-400 UV/VIS spectrophotometer.

2.21. Enzyme-product binding

As reaction intermediates were not detected by GC, the following experiments were performed to examine enzyme-product binding. Analyses were carried out using a Tenax packed column.

2.21.1. Protein-catechol binding

The following protein dissociation experiment was carried out to check for irreversible protein-catechol bond formation.

1. Samples were dispensed into Eppendorfs and microfuged for 30 seconds.
2. 200 μ l of the supernatants were removed into clean Eppendorfs and boiled for 5 minutes to denature the protein.

3. Five volumes of acetone were added to precipitate the protein.
4. After 45 minutes (a white precipitate would indicate protein dissociated from catechol) samples were respun in a microfuge.
5. The supernatant was removed and assayed on GC.

2.21.2. Protein-quinone binding

The following experiment was performed to check for protein-quinone binding.

1. An aerated bovine serum albumin (BSA) sample was mixed with quinone and analyzed on GC at time 0, 2 and 4 hours.
2. An aerated *A. bisporus* crude tyrosinase sample was incubated with quinone and analyzed on GC over time.
3. An aerated BSA sample, aerated *A. bisporus* tyrosinase sample and quinone were incubated together and analyzed on GC over time.

CHAPTER 3

SCREENING MICROORGANISMS FOR TYROSINASE

Summary A range of fungi and bacteria were screened for growth on phenolic agar plates and the production of polyphenolases under a variety of enzyme-inducing conditions. Three promising enzyme sources were selected and phenol removal efficiencies assessed in aeration experiments. Highest phenol removal was by the unidentified bacterium AECI culture no. 26.

3.1. INTRODUCTION

Aunstrup (1977) has identified a number of criteria to be considered when selecting sources for the production of microbial products. These include factors such as rapid and easy growth on comparatively cheap and simple nutrients without the use of inducers. A high yield of enzyme should be obtained in a form that is easy to isolate, purify and concentrate without the formation of toxic or immunogenic metabolites. The organism should have stable genetic and physiological characteristics and be readily acceptable to the food and drug authorities (Cheetham, 1986). Undesirable properties include odour, colour and slime formation, harmful metabolite production and unnecessary enzyme activity (Aunstrup, 1977).

The requirements for the finished product are safety, stability and reliability. A safe enzyme should have low allergenic potential and be free from toxic materials and harmful microorganisms. A high quality enzyme product should have a standardized enzyme activity and be easy to handle and use. Odour and other undesirable properties should be absent. Reliability implies enzyme stability during storage and resistance to microbial attack. High purity is not usually a requirement as it may be accompanied by reduced stability under storage and application conditions (Aunstrup, 1977). Only a few enzymes are marketed in pure or nearly pure form, mostly for research or analytical purposes (Kirk and Othmer, 1980).

While *Agaricus bisporus* is the best known producer of the phenol-oxidizing enzyme tyrosinase, which is produced by the fruiting body, it is not economically viable to produce a commercial enzyme from this source as the production of mushrooms is both land- and labour-intensive. A more practical source of the enzyme would be needed if it was to find application in an area such as effluent treatment. As polyphenolases are produced by a wide range of microorganisms, a screening programme was initiated for alternative producers of the enzyme system.

The design and operation of appropriate screening techniques has long been recognized as a worthwhile first step in the identification of suitable strains for the production of microbial products (Aunstrup, 1977). It is essential to correctly identify the characteristics to be selected for during screening. Characteristics desired in this screening procedure include the ability of selected microorganisms to grow in liquid culture without prior optimization, and the production of enzymes easily extractable by simple methods. Enzyme activities must be comparable to that of *A. bisporus* tyrosinase.

The screening programme designed encompassed a number of stages, with the final phase comprising a simulated phenol removal experiment during which phenol concentrations were monitored and removal efficiencies of selected enzyme extracts determined. The various phases of the screening programme are outlined below, together with references to the methods used.

1. Selection of microorganisms - section 2.1 and appendix 1.
2. Growth on solid substrate (plate tests) - section 2.2, appendices 2-4.
3. Enzyme production in shaker flasks - section 2.3.1, appendix 3.
4. Enzyme induction studies - section 2.4.1.
5. Enzyme extraction by ultrasonication - section 2.8.1.
6. Spot tests - section 2.10.
7. Enzyme assays - section 2.11.1.
8. Protein determinations and zymogram analysis - sections 2.12.1 and 2.13, appendices 6 and 7 respectively.
9. Phenol removal studies - section 2.18.
10. Product analysis - section 2.20.

A crude extract of *A. bisporus* tyrosinase was used as positive control when needed.

Given the complex requirements for enzyme production, each microbial system needs to be optimized to estimate best possible performance. This could clearly not be done in each case and organisms therefore had to perform under similar liquid culture conditions.

3.2. RESULTS

3.2.1. Growth on solid substrate (plate tests)

Growth of selected microorganisms on glucose-phenol agar plates is indicated in tables 3.1 and 3.2. Organisms in table 3.1 are held in the culture collection of the Biochemistry and Microbiology Department at Rhodes University.

Of all cultures screened, 83.7% grew on 0.01% phenol agar plates; many (61%) growing abundantly. Growth on 0.1% and 1.0% phenol agar plates was 39.5% and 18.6% respectively. Very little growth was seen on all of the positive 1.0% phenol agar plates. A linear decrease in growth on increasing concentrations of phenol was observed.

Growth on 0.1% and 1.0% phenol agar was used as a preliminary evaluation of enzyme production based on the assumption that these cultures would have the necessary enzymes to assimilate or degrade phenol.

Table 3.1: Growth of bacterial and fungal cultures on glucose-phenol agar plates.

CULTURES	PHENOL-CONTAINING AGAR PLATES		
	0.01 % phenol	0.1 % phenol	1.0 % phenol
<i>Verticillium lateritium</i>	++	-	-
<i>Rhizopus stolonifer</i>	+++	-	-
<i>Syncephalastrum racemosum</i>	+++	-	-
<i>Thamnidium elegans</i>	-	-	-
<i>Trypospermum myrti</i>	++	-	-
<i>Schizophyllum commune</i>	++	+	-
<i>Stereum hirsutum</i>	-	-	-
<i>Sordaria fimicola</i>	+++	+++	+
<i>Alternaria alternata</i>	-	-	-
<i>Bacillus</i> sp.	+++	++	+
<i>Fusarium culmorum</i>	++	-	-
<i>Penicillium claviforme</i>	+	-	-
<i>Tricothecium roseum</i>	+	-	-
<i>Mucor hiemalis</i>	+++	-	-
<i>Neocosmospora vasinfecta</i>	+++	++	-
<i>Penicillium spinulosum</i>	+++	-	-
<i>Trichoderma viridae</i>	+++	-	-
<i>Sclerotium rolfsii</i>	++	-	-
<i>Microascus trigonosporus</i>	+	-	-
<i>Chaetomium elatum</i>	-	-	-
<i>Coriolus versicolor</i>	+	-	-
<i>Neurospora crassa</i>	+++	-	-
<i>Stachybotrus chartarum</i>	-	-	-
<i>Coprinus comatus</i>	++	+	-
<i>Botrytis cinerea</i>	+++	-	-

+++ : good growth
 ++ : fair growth
 + : little growth
 - : no growth

Table 3.2: Growth of environmental isolates on glucose-phenol agar plates.

ENVIRONMENTAL ISOLATES	PHENOL-CONTAINING AGAR PLATES		
	0.01% phenol	0.1% phenol	1.0% phenol
AECI culture no. 7	+++	+	+
AECI culture no. 26	+++	+	+
AECI culture no. 29	+++	+	+
AECI culture no. 32	+++	+	+
Hogsback culture no. 1	+++	+	+
Hogsback culture no. 2	-	-	-
Hogsback culture no. 3	+	-	-
Hogsback culture no. 4	+++	+	-
Hogsback culture no. 5	+	-	-
Hogsback culture no. 6	++	-	-
Hogsback culture no. 7	++	+	-
<i>Suillus bovinus</i> mycelial culture	+++	+	+
<i>Suillus bovinus</i> stipe culture	+++	+	-
<i>Suillus bovinus</i> cap culture	+++	-	-
<i>Suillus bovinus</i> gill culture	+++	+	-
<i>Termitomyces umkowaani</i> gill culture	+++	++	-
<i>Termitomyces umkowaani</i> stipe culture	+++	++	-
LURGI effluent	-	-	-

+++ : good growth
 ++ : fair growth
 + : little growth
 - : no growth

Tyrosinase production by the selected *Streptomyces* cultures was confirmed by the appearance of melanin on tyrosine-containing M3 agar (Plates 3.1 - 3.3). Due to the very specific growth requirements of the Streptomyces, results of enzyme induction and optimization studies are presented in chapter 4. The strong indications of enzyme production on plate assays is therefore further evaluated in chapter 4.



Plate 3.1: Growth of *S. glaucescens* on tyrosine-containing agar.

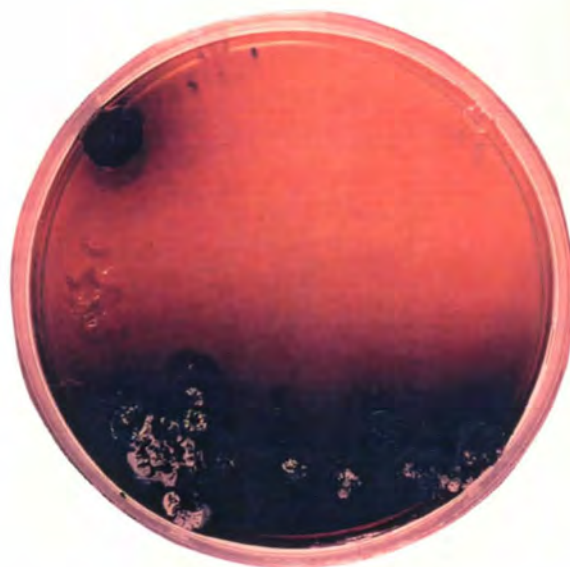


Plate 3.2: Growth of *S. antibioticus* on tyrosine-containing agar.

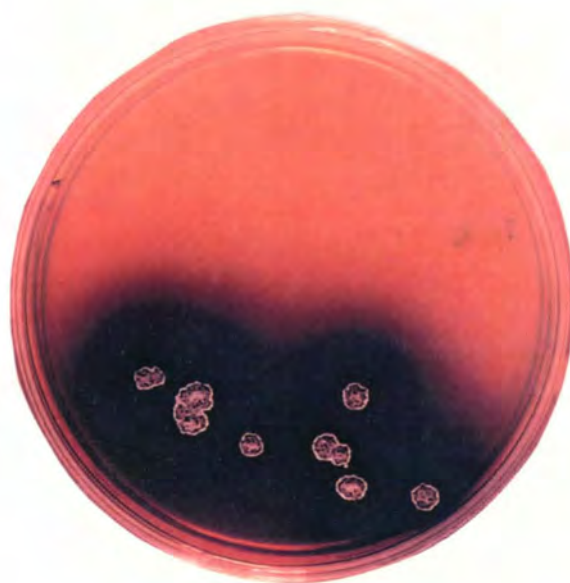


Plate 3.3: Growth of *S. lividans* (pIJ702) on tyrosine-containing agar.

3.2.2. Enzyme production in shaker-flasks

All shaker-flask experiments were carried out in glucose-phenol batch cultures and were performed in duplicate. Results are expressed as a mean value.

Intracellular enzyme was released by ultrasonication (section 2.8.1) and extracts assayed at 280nm (section 2.11.1) against two or all of the following substrates: L-tyrosine, phenol and a 1:1 mixture of the two phenolics (Table 3.3).

Table 3.3: Enzyme activity studies (activity expressed as units.ml⁻¹ extract).

CULTURES	EXTRACELLULAR EXTRACTS			INTRACELLULAR EXTRACTS		
	phenol	tyrosine	phenol/ tyrosine	phenol	tyrosine	phenol/ tyrosine
<i>S. commune</i>	-	0	615.4	-	769.2	0
<i>S. fimicola</i>	-	923.1	38.5	-	484.6	0
<i>Bacillus</i> sp.	-	146.2	1 253.8	-	0	0
<i>N. vasinfesta</i>	440.6	1 407.7	661.5	385.1	215.6	546.3
<i>N. crassa</i>	0	0	-	0	0	-
Hogsback no. 1	-	138.5	0	-	0	0
Hogsback no. 7	123.1	292.3	269.2	330.7	100.0	38.5
<i>T. umkowaani</i> stipe culture	84.6	192.3	84.6	300.0	115.4	692.3
<i>S. bovinus</i> mycelial culture	95.6	145.1	-	336.9	157.4	-
AECI no. 7	484.6	430.8	453.8	0	769.2	261.5
AECI no. 26	400.4	315.4	807.7	753.8	1 607.7	1084.6
AECI no. 29	692.3	238.4	146.1	0	423.1	269.2
AECI no. 32	1 007.7	830.7	138.5	400.0	323.1	700.0
Mushroom fruiting body	-	-	-	3 040.0	8 050.0	-

- : assay not carried out

Activity studies showed that none of the tyrosinase-producers evaluated had an enzyme activity comparable to that of the *A. bisporus* (mushroom) fruiting body. Only three organisms (23%) had an enzyme activity above 1 000 units.ml⁻¹ when assayed against phenol or tyrosine, i.e. *N. vasinfecta* (extracellular extract), AECI culture no. 26 (intracellular extract) and AECI culture no. 32 (extracellular extract). It is apparent that the ability of an enzyme to convert one phenolic compound does not necessarily guarantee catalytic ability on other phenolic compounds or on mixtures of substrates. Extracts also showed either high intra- or extracellular enzyme activity, but rarely both. Enzyme activity therefore depends on a combination of factors including source, locality and assay substrate. These results indicate a wide distribution of enzyme systems active on both phenol and tyrosine. Variability may be either endogenous or due to unoptimized systems.

3.2.3. Enzyme induction studies

Enzyme inducing systems were evaluated on promising organisms so as to eliminate the possible comparisons of unoptimized systems, i.e. ethylene induction (Table 3.4) and induction by the inclusion of metabolic inhibitors (Table 3.5). As inducers were added directly to fermentation broths, *A. bisporus* crude tyrosinase (extracted from fruiting bodies) was not included as a positive control.

Table 3.4: Enzyme induction by ethylene (enzyme activity expressed as units.ml⁻¹ extract).

CULTURES	EXTRACELLULAR EXTRACTS		INTRACELLULAR EXTRACTS	
	tyrosine	phenol/tyrosine	tyrosine	phenol/tyrosine
<i>N. vasinfecta</i>	407.7	230.7	0	130.7
<i>S. commune</i>	169.2	123.1	0	0
<i>S. fimicola</i>	923.1	146.2	77.0	277.0
<i>Bacillus</i> sp.	423.1	107.7	61.5	0
Hogsback no.1	592.3	69.2	0	0

Ethylene was evaluated as an enzyme inducer due to its known hormonal affect on plants (Bidwell, 1979) and the relationship between ethylene production and fruiting of cultivated *A. bisporus* (Wood and Hammond, 1977). Results in table 3.4 indicated that ethylene

appeared to repress, not induce, tyrosinase production in all cultures except Hogsback culture no. 1, where a slight increase in extracellular enzyme production was noted.

Due to the labour-intensive nature of the screening programme, single substrate assays were selected for subsequent studies. Tyrosine was used as the monophenolic substrate.

Table 3.5: Enzyme induction by the inclusion of metabolic inhibitors (extracts assayed against tyrosine and activities expressed as units.ml⁻¹ extract).

CULTURES	EXTRACELLULAR EXTRACTS			INTRACELLULAR EXTRACTS		
	SA	OT	IAA	SA	OT	IAA
<i>N. vasinfecta</i>	161.5	0	107.7	130.7	0	207.7
<i>S. commune</i>	-	0	69.2	-	653.8	361.5
Hogsback no. 1	0	6 246.1	161.5	230.7	53.8	261.6

SA: sodium arsenate
OT: oxytetracycline
IAA: iodoacetic acid
-: inhibitor not included

Tyrosinase production by Hogsback culture no. 1, particularly that of the extracellular extract, was notably increased by the inclusion of oxytetracycline to culture media. No other organisms showed any increase in enzyme production.

3.2.4. Enzyme detection systems

A number of assay systems, i.e. spot tests (section 2.10), enzyme assays (section 2.11.1) and zymograms (section 2.13), were evaluated for their convenience as enzyme-detection methods in order to reduce the labour-intensive nature of screening.

3.2.4.1. Spot tests

A colour reaction between enzyme extract and substrate gave a visual preliminary indication of tyrosinase presence. *A. bisporus* crude enzyme extract gave a bright brownish-red positive reaction against p-cresol, whereas controls showed no colour change.

Only the intracellular extract of culture AECI culture no. 26 showed a slight colour change. Enzyme production by this culture was also detected on assay. Spot tests did not appear to be effective at detecting low enzyme concentrations.

3.2.4.2. Enzyme assays

Enzyme assays were used throughout the screening procedure as a reliable indicator of tyrosinase presence and activity. All assay results were graphically represented and enzyme activities of positive assays calculated. An increase in absorbance at 280nm (associated with a colour change from yellow to orange) was indicative of a positive assay result i.e. tyrosinase production. Figure 3.1 is an example of a positive enzyme assay.

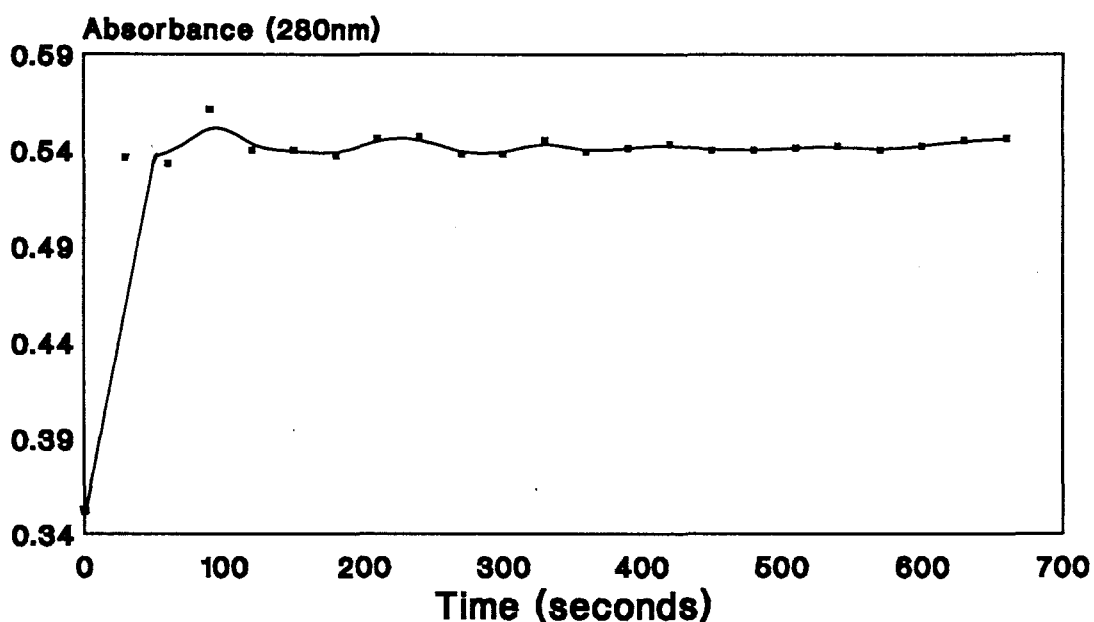


Figure 3.1: A positive L-tyrosine enzyme assay result.

3.2.4.3. Zymogram analysis

As enzyme assays appear to be of limited sensitivity, zymograms were evaluated for the rapid detection of enzyme production. Zymograms were developed against concentrated substrates as the reaction takes place at the enzyme/substrate interface along the surface

of the gel. Protein determinations (section 2.12.1) were carried out on selected extracts prior to zymogram analysis (Table 3.6).

Table 3.6: Protein determinations of extracted samples.

ENZYME EXTRACTS	PROTEIN CONCENTRATION (mg.ml ⁻¹)
<i>C. versicolor</i> - extracellular	1.47
<i>N. crassa</i> - extracellular	2.28
Hogsback no. 1 - extracellular	0.93
Hogsback no. 1 - intracellular	0.39
Hogsback no. 1 + OT - extracellular	0.68
Hogsback no. 1 + OT - intracellular	1.98
Hogsback no. 1 + eth - extracellular	0.15
Hogsback no. 1 + eth - intracellular	2.07
AECI culture no. 26 - intracellular	1.94
AECI culture no. 32 - extracellular	0.32
AECI culture no. 32 - intracellular	1.11
AECI culture no. 29 - extracellular	3.06
AECI culture no. 29 - intracellular	2.12
AECI culture no. 7 - extracellular	3.40
AECI culture no. 7 - intracellular	2.78
<i>A. bisporus</i> crude tyrosinase	3.75

OT : oxytetracycline

eth : ethylene

A. bisporus tyrosinase was present in all cases as two clearly defined protein areas. Only one other extract showed any indication of tyrosinase production, i.e. AECI culture no. 26 (intracellular extract) (Plate 3.4, lane 1). This result is consistent with spot test results.

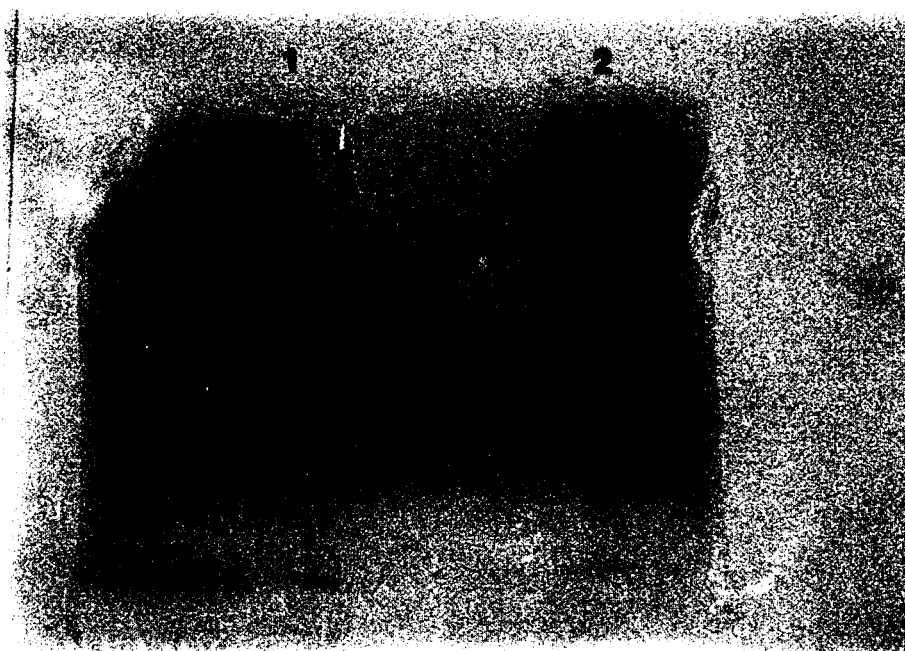


Plate 3.4: Zymogram developed against L-tyrosine.

Lane 1: AECI culture no. 26, intracellular extract.

Lane 2: Crude *A. bisporus* tyrosinase.

This system presents a useful rapid screening method for microorganisms producing appreciable quantities of tyrosinase. It also shows the correlation between enzyme production and protein concentration, and the number of proteins present. This method also differentiates between stable proteins which do not degrade on migration and unstable protein assemblies.

3.2.5. Enzyme production over time

This study was undertaken to identify periods of maximum tyrosinase production. *N. vasinfesta* was selected for this study based on its potential enzyme production observed in initial activity studies.

The production of tyrosinase over a 10 day period was determined by daily enzyme assays (Figure 3.2). In both the case of intra- and extracellular tyrosinase production, enzyme levels peak at days 1-2 and 7-8, implicating these as optimum harvesting periods.

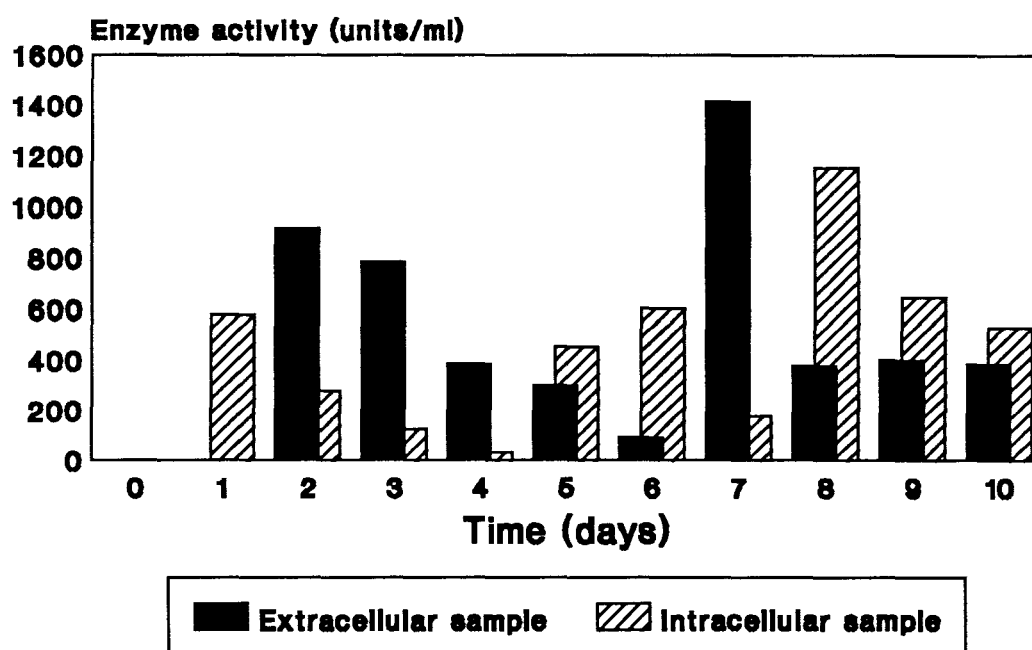


Figure 3.2: A profile of tyrosinase production by *N. vasinfecta*.

3.2.6. Enzyme inhibition studies

Studies on reaction inactivation due to inhibitory concentrations of enzyme and/or substrate were undertaken so as to identify factors which might adversely effect the efficiency of the enzyme reaction. This study was carried out using *N. vasinfecta* extracellular enzyme extract. Results are expressed in table 3.7.

Table 3.7: An examination of enzyme inhibition (results expressed as units of activity.ml⁻¹ extract).

ASSAY CONDITIONS	ASSAY SUBSTRATE	
	tyrosine	phenol
1x enzyme, 1x substrate	1 407.7	440.6
2x enzyme, 1x substrate	2 908.7 (2.07x increase)	1 782.0 (4.04x increase)
1x enzyme, 2x substrate	553.8 (2.54x decrease)	661.5 (1.5x increase)

These results indicate that the inactivation of enzyme activity is substrate, not enzyme, controlled. Enzyme concentration is positively correlated to enzyme activity. The nature of the substrate appears to influence the extent of reaction inactivation, e.g. tyrosine as substrate greatly reduced enzyme activity.

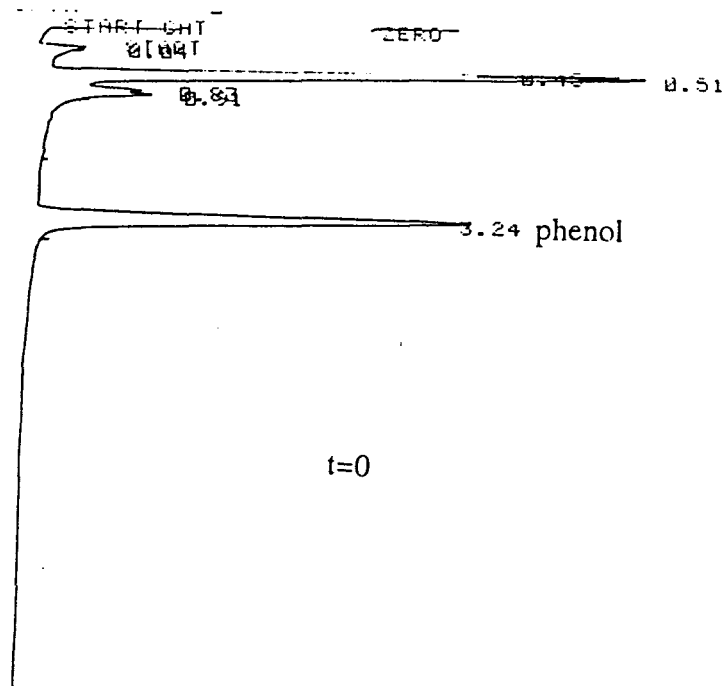
3.2.7. Phenol removal efficiencies and product analysis

A phenol removal assay was designed to evaluate enzyme function against a simulated phenol effluent. A number of cultures were selected for these investigations based on results of the screening programme. Removal efficiencies were determined against phenol as it is a common component of industrial effluents.

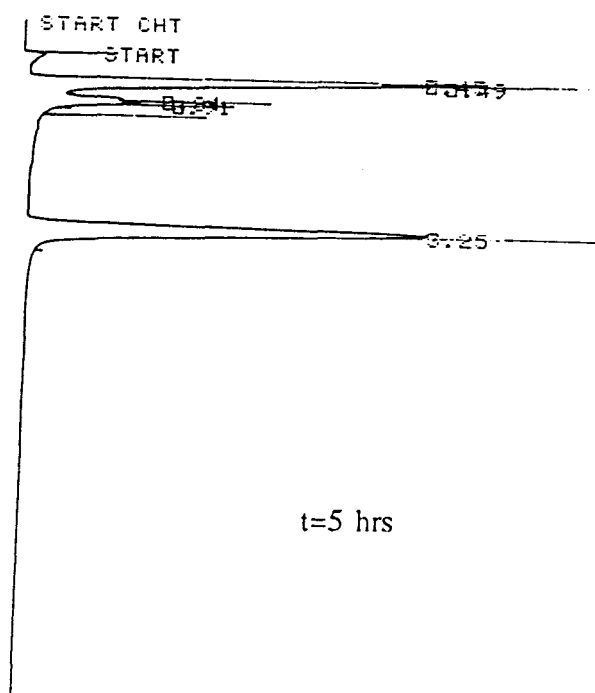
Although HPLC successfully separated phenolic standards using a 5-80% acetonitrile:water gradient with the detector wavelength set at 254 nm, gas chromatography (GC) was selected as the quickest and most efficient method for analyzing large numbers of samples. All samples were therefore analyzed by GC using a Tenax column (section 2.20.2.1).

3.2.7.1. Hogsback culture no. 1

The phenol removal efficiency of Hogsback no. 1 extracellular tyrosinase, induced by the metabolic inhibitor oxytetracycline, was determined (Figures 3.3 and 3.4). No colour changes and no reduction in phenol was seen. The activity seen against tyrosine on assay does not appear to extend to other monophenolic compounds. A pattern of fluctuating phenol concentrations was detected throughout the experiment (Figure 3.4).



Area = 12 060



Area = 11 480

Figure 3.3: Phenol removal efficiency of Hogsback culture no. 1 (extracellular extract, tyrosinase induced by oxytetracycline) as monitored by GC from 0 to 5 hours.

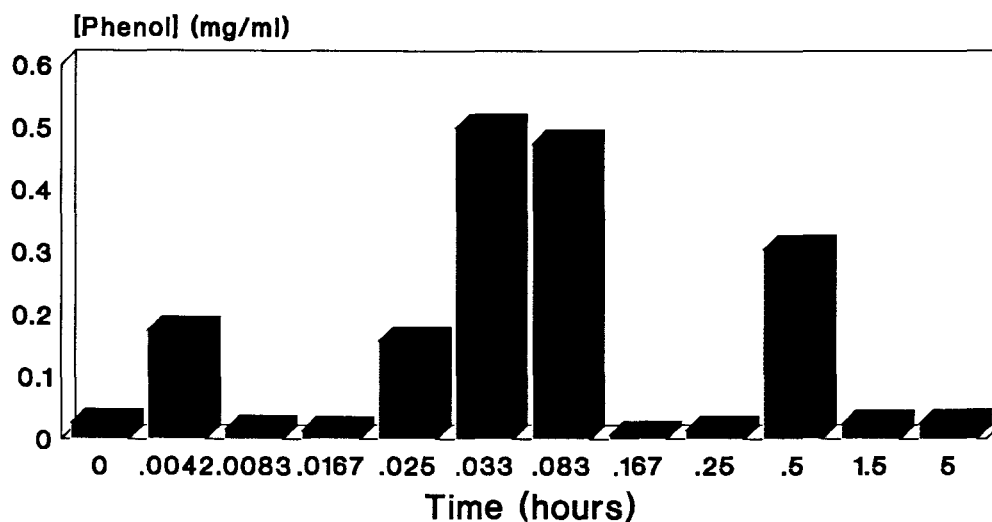
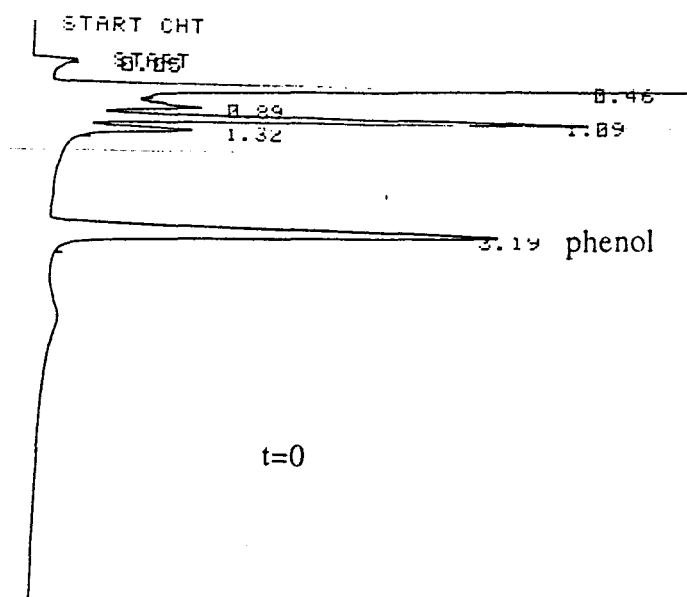


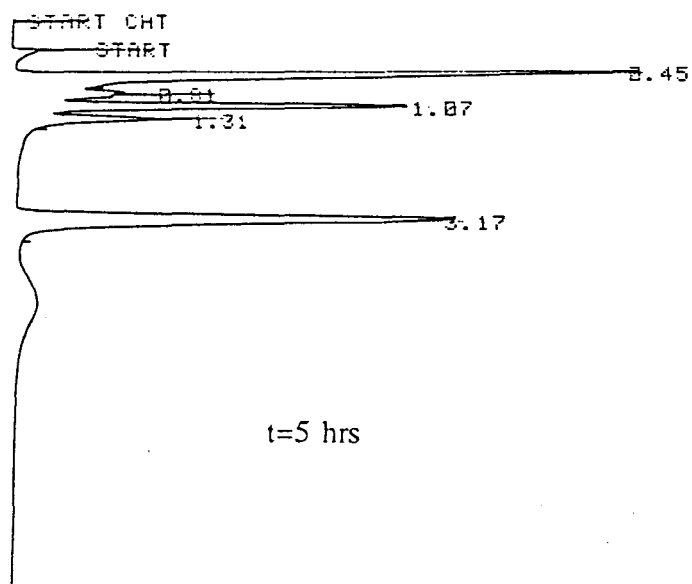
Figure 3.4: Phenol removal efficiency of Hogsback culture no. 1 (extracellular extract) induced by oxytetracycline.

3.2.7.2. AECI culture no. 32

Figures 3.5 and 3.6 show the phenol removal efficiency of AECI culture no. 32 (extracellular extract). A slight colour change from light to darker yellow was seen during the aeration experiment. No phenol removal was noted. A slight fluctuation in phenol levels was seen (Figure 3.6).



Area = 12 260



Area = 12 630

Figure 3.5: Phenol removal efficiency of AECI culture no. 32 (extracellular extract) as monitored by GC from 0 to 5 hours.

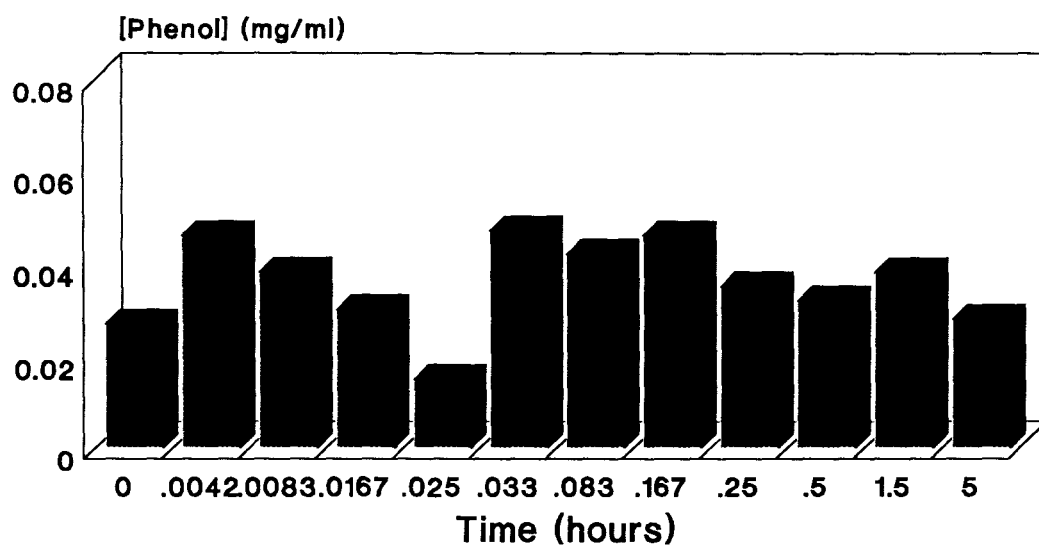


Figure 3.6: Phenol removal efficiency of AECI culture no. 32 (extracellular extract).

3.2.7.3. AECI culture no. 26

No colour change was seen during the phenol removal experiment using AECI culture no. 26 (intracellular extract). Fluctuating phenol concentrations were again noted with a 46.4% reduction in phenol after 5 hours (Figures 3.7 and 3.8). These results imply that colour changes alone are not necessarily reliable indicators of enzyme presence.

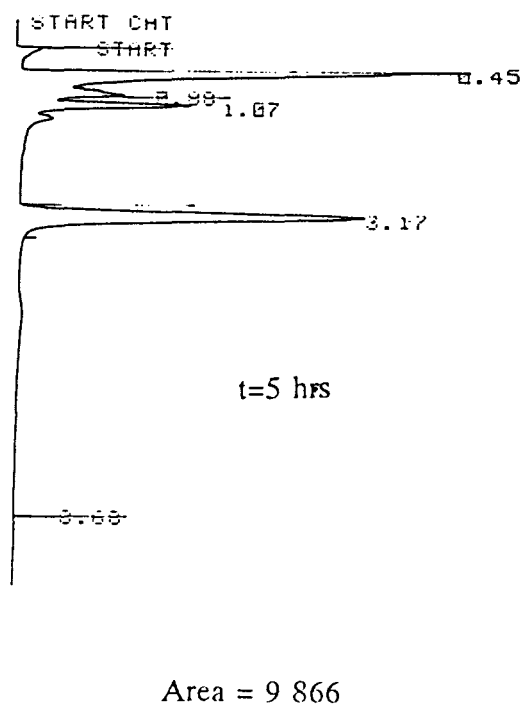
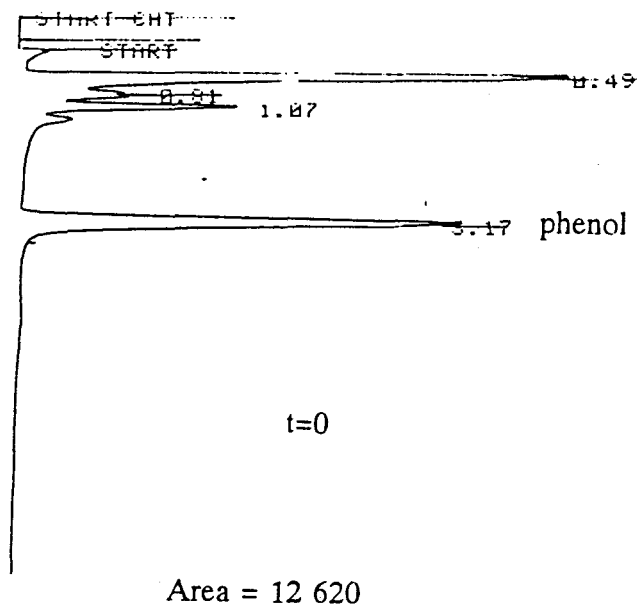


Figure 3.7: Phenol removal efficiency of AECI culture no. 26 (intracellular extract) as monitored by GC from 0 to 5 hours.

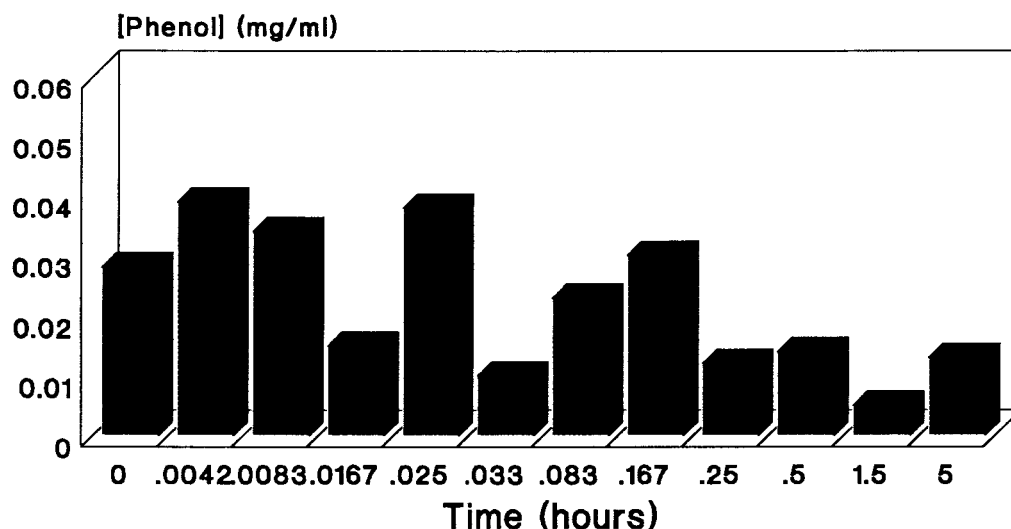


Figure 3.8: Phenol removal efficiency of AECI culture no. 26 (intracellular extract).

High removal efficiencies were recorded for *A. bisporus* crude tyrosinase; results are presented in chapter 5.

3.3. DISCUSSION

Before the screening programme could be implemented, it was essential to define the limits of the search. As seen in Chapter 1, polyphenol oxidases could present a practical method for the degradation of phenolic wastes present in effluents. As different methods and assay systems exist for the identification of the two polyphenolase enzymes, tyrosinase and laccase, it became necessary to restrict the search to one of these enzymes. Tyrosinase was selected as it oxidizes both monophenols and o-dihydroxyphenols. In 1984 Atlow *et al.* reported that tyrosinase was capable of co-precipitating substituted phenols and other pollutants, an important property when dealing with heterogeneous phenolic effluents. As bound phenols are more accessible to laccase, the ideal enzyme source would be one producing both laccase and tyrosinase, thereby extending dephenolization to include the p-dihydroxyphenols.

A simple monophenolic compound, phenol, was incorporated into agar medium and a wide range of fungi and some bacteria were screened for growth on phenol and the presence of polyphenolases. As laccase and tyrosinase co-exist in many sources, the search was not confined to brown-rot fungi. Atypical tyrosinase-producing white-rots have also been identified (Käärik, 1965).

3.3.1. Growth on solid substrate (plate tests)

The growth of microorganisms on specific solid substrates is widely employed as the first stage of a screening programme, e.g. a number of simple plate tests exist for the direct visualization of lignin degradation by microorganisms; positive results indicate the presence of polyphenol oxidases and/or peroxidase. White-rot fungi (generally laccase producers) show deeply coloured zones around mycelia grown on agar containing certain complex phenolic compounds (Sundman and Näse, 1971).

The development of colour - either of the mycelia or of the medium directly surrounding the mycelia - is assumed to be due to the formation of polyphenolic compounds if the substrate is phenol, or melanin when tyrosine is included in the agar medium. Colour reactions therefore imply that polyphenolases are being produced.

There are a number of requirements for a test substrate to be effective (Sjöblad and Bollag, 1981):

1. The test chemical must be specific for one oxidase, with one oxidation product formed;
2. The substrate should not autooxidize readily and not be oxidized by contaminating chemicals;
3. When whole organisms are to be used, a suitable substrate should be non-toxic to the test organism.

An important function of this agar plate method was to determine microorganism sensitivity to phenol toxicity. According to Käärik (1965) brown rots are more susceptible to damage by phenolic compounds. Most organisms screened showed little sensitivity to

low phenol levels. Phenol also satisfied the second requirement as it does not autooxidize rapidly.

It seems a general phenomenon that when microorganisms are exposed to persistent contaminants, they develop enzyme systems to degrade these pollutants. Phenol was therefore chosen as a monophenolic test substrate in an attempt to induce tyrosinase production. This selection was also based on the composition of industrial effluents. However, phenol specificity for tyrosinase induction may be questionable as it is also the inducer of the enzymes of the meta- pathway for the assimilation of phenol by microorganisms. This pathway is not confined to bacteria. Fungi which have it include *Penicillium* and *Aspergillus* (Dart and Stretton, 1980). Käärrik (1965) also reported that phenol is not a reagent specific to laccase or tyrosinase and is often not oxidized when present. This variable response to phenol is demonstrated by the results of this study.

When a microorganism grows on phenol, one or both of two processes may take place. Degradation (dissimilation), where phenol is changed to less toxic end-products, i.e. energy metabolism, or synthesis (assimilation), where it is incorporated into the cell substance i.e. anabolism. Residence times and intensity of aeration determine whether degradation or synthesis predominate. Dissimilation or degradation is important in waste treatment, while assimilation is normally restricted to low phenol concentrations (Martinetz, 1986). Phenol assimilation could therefore be responsible for the abundant growth of most organisms on 0.01% phenol agar plates. Beveridge and Tall (1969) reported that phenol is degraded readily at low concentrations by many microorganisms. A marked decrease in growth on 0.1% and 1.0% phenol may implicate the dominance of phenol degradation, or a tolerance for phenol in the growth medium.

Studies on wood-degrading Basidiomycetes showed that the ability or inability to oxidize phenol in agar media could not be correlated with the ability or inability to decompose lignin (Sundman and Näse, 1971). It may therefore not be feasible to correlate growth on phenol-containing agar with the presence of phenol-degrading enzymes. Subsequently, growth on 0.01% phenol agar may not be indicative of tyrosinase presence. Growth and/or the formation of brown colonies on 0.1% and 1.0% phenol agar seems a more feasible indication of enzyme production.

Tyrosinase and laccase production is most frequently found amongst fungi belonging to the Agaricaceae and to the genera *Peniophora* and *Stereum*. They are also found in limited Polyporaceae species (Käärik, 1965). Of the fungi from the culture collection which grew on 0.1% phenol agar, half were of the class Basidiomycetes, order Agaricales; *S. commune* and *C. comatus* are of the family Agaricaceae (also *A. bisporus*) and *S. bovinus* of the family Boletaceae. *S. fimicola* and *N. vasinfecta* are of the class Ascomycetes and *T. umkowaani* is of the order Agaricales, family Tricholomataceae (Alexopoulos, 1962).

There is still some question as to whether growth on 0.1% phenol agar does indicate tyrosinase production. Although *S. commune* grew on 0.1% phenol agar, the literature reports it as a white-rot laccase-producer which does not produce any tyrosinase (Phillips and Leonard, 1976; De Vries *et al.*, 1986). Other tyrosinase-producing fungi that did not grow on 0.1% phenol agar included *N. crassa* (Horowitz, 1965; Horowitz *et al.*, 1970) and *S. rolfsii* (Sjogblad and Bollag, 1981). Tyrosinase is however, only produced under particular conditions in *N. crassa*. The extreme variability of tyrosinase production by different organisms has been widely reported in the literature and is confirmed by the results of this study.

Käärik (1965) reported that conflicting results for a particular fungal species may be explained by the variability of the oxidation capacity between different strains of the same fungus. Dissimilar results are also obtained using different methods, e.g. tyrosinase is induced in a particular strain of *N. crassa* by inhibiting growth, either by starvation in a pH 6 phosphate buffer (Urey and Horowitz, 1967) or by using certain toxic substances (Horowitz, 1965). Laccase production can also be induced in *N. crassa*, but by the inclusion of protein synthesis inhibitors (Froehner and Eriksson, 1974). Enzyme production is also influenced by internal factors associated with development e.g. age of hyphae (Marr *et al.*, 1986).

A number of fungi are reported to produce both laccase and tyrosinase in varying amounts and under different conditions e.g. *A. nidulans* (Bull and Carter, 1973; Birse and Clutterbuck, 1990), *N. crassa*, *Rhizoctonia praticola* (Sjogblad *et al.*, 1976; Leonowicz *et al.*, 1984; Bollag *et al.*, 1988), *Lepista*, *Russula* and *Lactarius* species (Matsubara and

Iwasaki, 1972). Unfortunately it is very difficult to optimize the simultaneous production of tyrosinase and laccase.

3.3.2. Enzyme production in glucose-phenol shaker-flasks

Although *N. crassa* did not grow on 0.1% and 1.0% phenol agar plates, it was passaged into liquid culture due to numerous reports of its tyrosinase-producing ability. Other microorganisms had been selected due to good preliminary indications of enzyme production on phenol agar plates.

Only 23% of cultures grown in glucose-phenol liquid media yielded an enzyme activity above 1 000 units.ml⁻¹ extract, that is approximately 13% of *A. bisporus* crude tyrosinase activity. The low activity of *S. bovinus* tyrosinase was consistent with results published by Ramstedt and Söderhäll (1983). Research and literature results were also consistent for *S. commune* (Phillips and Leonard, 1976; De Vries *et al.*, 1986). A number of environmental isolates gave promising results.

In some cases enzyme production in liquid media was vastly different from preliminary indications on solid substrate. Käärrik (1965) suggested that phenol oxidases may be lost during the cultivation of mycelia. This may also explain why some strains of a particular fungus may differ from others in their reaction capacity. Factors influencing results include culture media, aeration, temperature, pH, age of the mycelium and variations between different isolates of a fungus species. Fahraeus (1952), cited in Schanel (1966), stated that the capacity to form and secrete enzymes is associated with the cultivation conditions of the fungus.

It is plausible that the composition of culture media would affect enzyme production. With the exception of glucose concentration, media composition is similar to that used by Dion (1952) for the production of polyphenol oxidase in the fungus *Polyporus versicolor*. Dion used 30 g.l⁻¹ glucose, whereas this study uses only 5 g.l⁻¹. It is possible that the phenol included as inducer was utilized by the microorganisms as carbon source due to glucose exhaustion. Assimilation would therefore predominate over dissimilation and tyrosinase production would be low or absent. However, excess glucose can be detrimental. Bollag

and Leonowicz (1984) reported the inhibition of *Trametes versicolor* laccase production by excess glucose. It is therefore essential to maintain a balance between the prevention of enzyme inhibition and glucose exhaustion.

In 1990 Spånnig and Neujahr reported that the role of phenol in culture media changed from carbon source to inducer on the inclusion of glucose. It therefore seems unlikely that phenol assimilation plays a large role in glucose-phenol liquid media cultures.

It is interesting to note that Dion (1952) had not included any copper or enzyme inducer into *P. versicolor* liquid growth medium. Laccase and tyrosinase production has repeatedly been reported to be inducible (Käärik, 1965; Rähä and Sundman, 1975). In 1982, however, Leonowicz and Malinowska discovered inducible and constitutive forms of the laccase produced by *Pholiota mutabilis*. In 1938 Kubowitz (cited in Matthew and Parpia, 1971) established that tyrosinase contained copper essential for activity. The inclusion of copper into liquid media should therefore improve enzyme production. All these factors stress the absolute variability of conditions optimum for enzyme production.

Distinct variations appear to exist between tyrosinase production in liquid media and on a solid substrate. A number of factors influence enzyme yield, such as the availability of the phenol inducer and substrate to the microbial cell. As tyrosinase has been widely reported as being mainly intracellular (Käärik, 1965; Lyr and Luthardt, 1965), the successful transport of compounds across the cell membrane is essential. Most aromatic compounds in culture media can gain access to the intracellular enzyme by simple diffusion; specific transport systems have also been reported (Reineke, 1984). Phenol is readily soluble in the culture medium.

Extracellular tyrosinase production may be due to one of the following systems (Schanel *et al.*, 1971):

1. Secretion or "leaking out" of the internal enzyme into the culture medium without changes in molecular properties;
2. Secretion of an enzyme synthesized especially for extracellular action;

3. Secretion of a primarily internal enzyme with certain properties changed before or during transport through the cell membrane.

3.3.3. Enzyme induction studies

3.3.3.1. Enzyme induction by ethylene

Ethylene is widely-known for its hormonal effects on plants, where a wide range of responses are elicited, e.g. in leaves where it promotes senescence and in fruit where it fosters ripening. It also causes epinasty, geotropism and abscission, and is usually classed as an inhibitory hormone. Ethylene does not appear to have a pronounced effect on any specific biochemical reaction, but may stimulate protein production during ripening (Bidwell, 1979).

Studies carried out by Turner *et al.* (1975) and Wood and Hammond (1977) found a correlation between the fruiting and ethylene production of cultivated *A. bisporus*. Maximum ethylene production was noted at the expansion stage of the mushroom fruiting body i.e. the stage associated with maximum tyrosinase production. Ethylene production occurred within the compost and was not produced by the fruiting body itself. Reduced laccase levels were noted in the compost during ethylene production, indicating possible mycelial involvement. It was proposed that the mycelium-compost complex produces a burst of ethylene coinciding with the development of the mushroom fruiting body. It is not known how or why ethylene is produced, but it is not improbable that its production may be consistent with tyrosinase production in the fruiting body.

Hogsback culture no. 1 was included in this study as growth was seen on 1.0% phenol agar. As very little enzyme was produced on substrate induction in liquid media, enzyme induction by ethylene was investigated. It was the only culture that showed any change in enzyme production on the addition of ethylene; a slight increase was seen in extracellular enzyme production.

The results of this induction experiment indicated that the presence of ethylene alone does not stimulate tyrosinase production. A finer, more complex control system must exist between mushroom mycelia, the fruiting body and laccase and tyrosinase enzyme systems.

3.3.3.2. Enzyme induction by the inclusion of metabolic inhibitors

In some fungi tyrosinase is only present under special cultural conditions, but can be induced by the addition of several chemicals which are not normally substrates for the enzyme i.e. metabolic inhibitors (Lyr and Luthardt, 1965). Actinomycin D studies on tyrosinase induction by pentachlorophenol (PCP) in *Fomes marginatus*, showed that enzyme induction appeared to be bound to an active protein metabolism. A disturbance of the oxidative metabolism, especially ATP formation, led to enzyme induction. Lyr and Luthardt (1965) proposed that the enzyme induction was due to the liberation of an internal inducer or the abolition of a metabolic repressor. The induction of tyrosinase by this method will therefore vary according to species as it appears to be regulated by the condition of the metabolism rather than the metabolic inhibitor employed.

The concentration of the inhibitor is important as high concentrations may be toxic to the organism or inhibit enzyme formation. However, cases have been reported of enzyme induction only after growth has been inhibited, e.g. all phenols which induce laccase production of *Fomes annosus* by more than two-fold are toxic or at least inhibit growth (Haars *et al.*, 1981).

The metabolic inhibitors employed in this study are all included at low concentrations i.e. approximately 10^{-4} M. Sodium arsenate and iodoacetic acid show little influence on enzyme production, but oxytetracycline has a much stronger effect (Table 3.4). Oxytetracycline is a tetracycline antibiotic which inhibits binding of aminoacyl-t RNAs to ribosomes (Stanier *et al.*, 1976). It totally inhibits enzyme production in *N. vasinfesta*, but induces significant extracellular enzyme activity in Hogsback culture no. 1, despite this organism's relatively low protein content. This result may suggest some effect on the cell wall of the organism which would facilitate enzyme secretion out of the cell. Sodium arsenate and iodoacetic acid also have some inducing effect on tyrosinase production by the latter organism. The metabolism of Hogsback no. 1 appears to be receptive to enzyme induction. This proposal

is borne out by ethylene-inducing results in section 3.2.3. In both cases the extracellular enzyme extract shows some increase in activity on induction.

3.3.4. Enzyme detection systems

3.3.4.1. Spot tests

This method presents a useful option for the quick testing of extracted samples for the presence of tyrosinase. The effect of phenolic substances on growing organisms is eliminated (Käärik, 1965). Advantages of this method include the use of controls and enzyme-specific reagents and the visual indications of enzyme presence (Marr *et al.*, 1986).

The only strong positive reaction was that of the positive control i.e. *A. bisporus* crude tyrosinase. AECI culture no. 26 (intracellular extract) also gave a slight colour reaction which correlates well with zymogram analysis. The negative reactions displayed by other extracts may be explained as follows. Firstly, a threshold enzyme activity is probably necessary for a positive reaction, suggesting the method may be insensitive. It is also possible that extracts did not contain tyrosinase or that the extracted enzyme was not tyrosinase. Other inherent factors which might interfere with results include the following (Marr *et al.*, 1986):

1. Autooxidation of the substrate;
2. Solvent reactions;
3. Sample pigments.

It is unlikely that any of these factors would affect the reaction. The aqueous solvent is harmless and substrate autooxidization is unlikely to take place within 15 minutes. Coloured pigments of the tyrosinase crude extract did not appear to interfere with test results.

In conclusion it appears that this method is not very sensitive for the preliminary assay of low enzyme concentrations.

3.3.4.2. Enzyme assays

The increase in absorbance at 280nm during a tyrosine enzyme assay is largely due to the tyrosine-DOPA conversion, with a slight contribution from the further oxidation of DOPA (Duckworth and Coleman, 1970). This assay system therefore follows the cresolase reaction catalyzed by, and specific to, tyrosinase.

In 1959 Frieden and Ottesen first reported that the tyrosine assay method was not satisfactory for crude enzyme fractions as many interfering substances (e.g. extraneous proteins, chromogenic impurities) show absorption in this range. This finding was supported by Matthew and Parpia in 1971. Tyrosinase also shows a strong absorbance maximum at 282nm. The sudden increase in absorbance seen on assay could therefore be indicative of enzyme presence, but not necessarily enzyme activity i.e. catalytically inactive enzyme.

Scott (1975) illustrates this assay pattern as rapid reaction kinetics symptomatic of tyrosinase. He states that the short (30-90 seconds) linear initial rate is due to rapid enzyme inactivation by spontaneous product formation. This hypothesis suggests that DOPA (catechol) as well as quinone bind to and inactivate the enzyme. Controls were used to discount any background absorbance not due to enzyme-substrate interaction.

3.3.4.3. Zymogram analysis

Results of zymogram analysis correlated well with those of other detection systems. Only AECI culture no. 26 (intracellular extract) showed any colour development (Plate 3.4). This extract also reacted positively on spot test analysis and showed the highest activity (excluding *A. bisporus* crude tyrosinase activity) on assay against L-tyrosine and phenol.

3.3.5. Enzyme production over time

Intracellular and extracellular tyrosinase production by *N. vasinfecta* was cyclical over a period of 10 days. The production of intra- and extracellular enzyme appeared to rise at days 1-2 and 7-8, suggesting maximum enzyme production at these times.

3.3.6. Enzyme inhibition studies

Results reported in section 3.2.6 clearly indicate that enzyme deactivation on assay is substrate controlled. "Reaction inactivation" of tyrosinase has been widely reported and is confirmed by this study.

The extent to which a reaction goes to completion is a function of the initial enzyme concentration. The concentration of the substrate is much greater than that of the enzyme and the rate of reaction is zero order with respect to the reactants i.e. it depends only on the concentration of the enzyme present. The extent of the reaction, however, is governed by the substrate concentration. The velocity of the reaction remains constant until nearly all the substrate has been consumed. The rate of the reaction now becomes dependent on the remaining substrate concentration and is first order with respect to it (Cheetham, 1986).

If the substrate concentration is above a certain limit, the enzyme (tyrosinase) is inactivated and oxidation stops. Monophenolic substrate molecules become covalently bonded to the enzyme during tyrosinase-catalyzed oxidation. This is the transition state complex (ES). If a high concentration of substrate molecules bind to the tyrosinase enzyme, it seems probable that the copper and oxygen attachment sites vital for catalytic activity may be masked, and the reaction stops. Scott (1975) has shown that at high substrate concentrations, tyrosinase activity or substrate oxidation stops long before all substrate is utilized. During the oxidation of tyrosine by mammalian tyrosinase extracted from hamster melanoma, oxidation will stop at substrate concentrations higher than approximately $8 \times 10^{-4}\text{M}$, i.e. tyrosine inhibits its own hydroxylation (Pomerantz, 1964). The adverse effect of high tyrosine concentrations on the enzyme reaction has been demonstrated by this study.

Excess substrate inhibition is a form of reversible inhibition caused by the formation of non-productive complexes. The formation of these complexes can be reduced by using lower substrate concentrations. Inhibition by substrate is less common than other forms of inhibition, and occurs at high substrate concentrations even though inhibition is not readily apparent and Michaelis-Menten kinetics are obeyed at low substrate concentrations. Enzyme activity evaluated in a batch culture assay may also give an underestimate of the

activity that can be achieved in a column reactor, if substrate inhibition occurs (Cheetham, 1986).

3.3.7. Phenol removal efficiencies

The following conditions were essential for enzymatic aerobic degradation of phenol:

1. An adequate supply of oxygen;
2. Maintenance of pH in the range 5-9 and the avoidance of sudden pH variations;
3. No enzyme inhibitors;
4. Optimal substrate concentration so as to prevent substrate inhibition of the enzyme. The phenol concentration must, however, be representative of phenolic effluents;
5. Maintenance of a constant temperature. Increasing the temperature will increase the rate of degradation, but lower the rate of oxygen supply and enzyme stability.

Unfortunately no reaction intermediates were identified on product analysis. This could be due to the speed at which the tyrosinase reaction takes place in aqueous systems. Catechol and o-quinone formation is spontaneous and irreversible. Consequently, the reaction process could not be verified. Changes in phenol concentration was followed on GC to give an indication of an enzyme's dephenolizing ability.

Hogsback culture no. 1 (extracellular extract) did not remove any phenol from the medium, but an unusual phenol pattern was seen throughout the reaction. This pattern was duplicated by reactions of AECl cultures no. 32 and 26 and seemed to indicate a sequential pattern of phenol binding and release. The formation of the transition state (enzyme-substrate) complex may be responsible for this unusual pattern. It is possible that the enzyme was partially or totally inhibited by the phenol substrate, but was attempting to bind the substrate in the enzyme-substrate complex formed prior to oxidation.

AECl culture no. 32 did not remove any phenol from the reaction medium. AECl culture no. 26 showed the most phenol removal over a 5 hour period, i.e. 46.4%. The result obtained in this experiment is a confirmation of phenol removal ability suggested by spot tests, enzyme assays and zymogram analysis. Although removal efficiency is substantially

lower than that of *A. bisporus* tyrosinase (Atlow *et al.* (1984) report phenol removal of up to 99%), it is a promising result.

As an organism's ability to produce a particular enzyme is associated with its cultivation conditions, the method by which organisms are screened is important. Published reports show that the induction of enzymes in different microorganisms occur under very different conditions. Optimum tyrosinase-producing conditions are therefore extremely variable. Instituting a screening programme which would satisfy all possible cultural requirements would be expensive and labour intensive. When a large number of organisms are to be screened, it is essential that a simple, effective screening system be adopted. Methods should be as close to the application methods as possible. One selection system was therefore employed for all organisms and certain assumptions concerning enzyme production had to be made. Microorganisms were evaluated under very limited culture conditions. The measurement of enzyme activity was also complicated by rapid inactivation of the enzyme, further polymerization of quinone products and the inhibition of activity at high substrate concentrations. Additional enzyme activities were not tested or accounted for.

A study of this nature therefore provides basic knowledge and new outlooks and ensures that results obtained can be applied, together with existing technologies, to the economic production of the desired commodity.

3.4. CONCLUSIONS

A number of conclusions can be drawn from the results obtained in this section of the study:

1. Dissimilatory and/or assimilatory processes may be responsible for growth on phenolic agar.
2. Growth and/or the formation of brown colonies on 0.1% and 1% phenol agar is assumed to be indicative of tyrosinase production.
3. Only 23% of microorganisms screened showed an enzyme activity above 1 000 units.ml⁻¹, i.e. 13% of *A. bisporus* crude tyrosinase activity.

6. Tyrosinase activity is substrate inhibited.
7. Spot tests appear to lack sensitivity for detecting low enzyme concentrations.
8. Phenol removal ability does not necessarily correlate with colour changes on phenolic agar or enzyme assay results.
9. AECI culture no. 26 showed a 46.4% phenol removal efficiency.
10. Although a number of tyrosinase producers were isolated during the screening programme, the Streptomyces were selected as the most viable alternative to *A. bisporus* as enzyme source.

CHAPTER 4

STREPTOMYCES AS A SOURCE OF TYROSINASE

Summary Three Streptomycetes were investigated for tyrosinase production in various culture media. pH 7.2 GYM medium induced the highest enzyme production in *S. antibioticus* and *S. glaucescens*, while *S. lividans* (pIJ702) showed good enzyme production in pH 7.6 MMT.

4.1. INTRODUCTION

Among the prokaryotes, a mycelial growth habit is confined to gram-positive bacteria and is characteristic of the Actinomycetes. In the proactinomycetes mycelial development is limited; specialized spores are not produced and reproduction is by mycelial fragmentation into short, rod-shaped cells. The euactinomycetes (group III) undergo development only in the mycelial state and reproduce through the formation of unicellular spores, differentiated either singly or in chains at the tip of the hyphae. The *Streptomyces* are the largest genera of the euactinomycetes. These microorganisms are abundant in soil and the characteristic odour of damp soil is due to volatile substances (geosmins) they produce. The primary degradative activity of actinomycetes is the solubilization of lignin (Ball *et al.*, 1989). Since the discovery of antibiotics as a secondary metabolite of these organisms in 1945, *Streptomyces* have acquired great economic importance (Stanier *et al.*, 1976).

Developmental cycle

The *Streptomyces* are characterized by the formation of a branching substrate mycelium interrupted by occasional cross-walls. Nutrient depletion leads to the formation of aerial mycelia as branches of the substrate mycelia. Differentiation into spore chains occur at the ends of the hyphae, while substrate mycelia undergo massive lysis. At maturity sheathed spores separate and detach from the hyphae. The internal structure and enzymes of the spores are very similar to those of the hyphae. Developmental events can be divided into four stages, viz:

- Stage 0: Vegetatively growing hyphae.
- Stage 1: Aerial hyphae assume characteristic coiling.
- Stage 2: The hyphal strand is divided into individual cells by the appearance of cross-walls. DNA replication takes place.
- Stage 3: New cell material is synthesized and cell walls thicken.
- Stage 4: Spores become rounded as outer hyphal walls disintegrate.

Spore germination takes place in three stages, i.e. activation, initiation and outgrowth. Physiological events associated with germination include an increase in the rate of endogenous respiration, an increase in ATP content and the initiation of RNA and protein biosynthesis.

All Streptomycetes are strict aerobes. Nutrition is simple and growth factors are not required. Biopolymers may be hydrolyzed by extracellular enzymes and used as carbon and energy sources (Dworkin, 1985).

Genetics

The genetics of *Streptomyces coelicolor* has been studied extensively, particularly by Hopwood and colleagues. By measuring the frequencies with which different donor genes were inherited together in multifactorial crosses, Hopwood constructed a genetic map for *S. coelicolor* (cited in Dworkin, 1985). All markers analyzed lay on a single circular linkage map (Stanier *et al.*, 1976). Approximately 100 genes have been recognized and mapped (including 12-13 morphological and developmental genes) (Dworkin, 1985) and about 29 genes have been sequenced to date (EMBL DNA Database, 1992).

Streptomyces DNA has a high G + C (guanidine + cytosine) content i.e. 63-72%. The genome is large in comparison to other prokaryotes e.g. *S. coelicolor* genome has a molecular weight of 7.1×10^9 daltons (Dworkin, 1985).

The production of tyrosinase and subsequent black melanin (eumelanin) formation has been reported for almost half the *Streptomyces* species isolated from natural habitats, including

S. antibioticus, *S. glaucescens* and *S. michiganensis* DSM 40015. Its function, however, is still unknown (Held and Kutzner, 1990).

Melanin production in tyrosinase-producing Streptomyces

The "tyrosinase reaction" of the actinomycetes was identified in 1908 by Lehmann and Sano (Skinner, 1938). *Streptomyces* tyrosinase is a monomeric metalloprotein of approximate molecular weight 30 000, consisting of 272 amino acids (Sugiyama *et al.*, 1990). It catalyzes the hydroxylation of tyrosine to L-DOPA and its subsequent dehydrogenation to dopaquinone. Dopaquinone is an unstable compound which further reacts to form melanin. Melanin is synthesized *in vivo* and forms an insoluble heteropolymer composed of 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid units. Due to the reactivity of quinones produced by tyrosine and DOPA oxidation, a variety of other compounds inside the cell can become incorporated into the precipitable pigment. Different chemical moieties can therefore act as chromophores in the final melanin heteropolymer. A variety of other effective substrates for *Streptomyces* tyrosinase can potentially be incorporated into the melanin polymer (della-Cioppa *et al.*, 1990). Melanin is only produced in the presence of tyrosine (Baumann *et al.*, 1976).

Melanin is therefore formed due to the expression of tyrosinase from the *mel* gene (della-Cioppa *et al.*, 1990). Tyrosinase extracted from *S. glaucescens* and *S. antibioticus* has two copper atoms in its active site (Katz and Betancourt, 1988; Gardner and Cadman, 1990), both which are necessary for the activity of the enzyme. The copper atoms are bound to two histidine residues in the active centre of the enzyme (Held and Kutzner, 1990). Lerch and Ettlinger (1972), Baumann *et al.* (1976) and Locci (1976), reported the presence of only one atom of cuprous copper per molecule of *S. glaucescens*.

Prokaryotes produce a single tyrosinase, unlike eukaryotes which produce a mixture of several isozymes (Lerch and Ettlinger, 1972).

Streptomyces antibioticus

The *mel* locus of *S. antibioticus* was originally isolated by Katz *et al.* (1983) and contains two open reading frames (ORFs) that encode a putative ORF438 protein ($M_r = 14\ 754$) and tyrosinase ($M_r = 30\ 612$). These are transcribed from the same promoter and both genes are required for high level melanin production. ORF438 has been shown to function as a *trans* activator of tyrosinase production (della-Cioppa *et al.*, 1990). These two ORFs are also known as *melC1* and *melC2* (Wu Lee *et al.*, 1988).

The structural gene for the inducible enzyme in *S. antibioticus* does not contain a signal sequence for secretion. However, putative signal (30 amino acids in length) (Bernan *et al.*, 1985) and attenuation sites are contained in ORF438, a 438-base pair nucleotide sequence located 5' to the structural gene (Gardner and Cadman, 1990). The ORF438 protein may therefore be involved in tyrosinase secretion, or it may function as a metallothionein-like protein that delivers copper to the apotyrosinase. Bernan *et al.* (1985) suggested that ORF438 protein might be a regulatory protein for the induction of tyrosinase or an accessory protein for the secretion of the enzyme. Betancourt *et al.* (1992) propose, however, that this sequence has a role in the phenotypic expression of tyrosinase. They suggest that the ORF438 gene product serves as a translocation protein for the export of tyrosinase. In the absence of the tyrosinase gene, ORF438 protein shows no detectable tyrosinase activity or melanin production. In the absence of ORF438 protein, clones with high levels of catalytically active tyrosinase were still poor melanin producers. This 1.55kb fragment of *S. antibioticus* DNA therefore appears to carry natural initiation signals for transcription and translation of the tyrosinase gene (Katz *et al.*, 1983). The existence of an ORF438 protein has not yet been confirmed *in vivo* (della-Cioppa *et al.*, 1990).

Sevcik (1957) reported a phenol oxidase of the laccase type in *S. antibioticus*. The presence of this enzyme appeared to be linked to the production of actinomycin, a quinone antibiotic.

Streptomyces glaucescens

S. glaucescens strain ETH 22794 has the highest tyrosinase activity of known melanin-positive Streptomyces. It produces a single intracellular cell-bound tyrosinase which is secreted into the culture medium, where melanin formation occurs (Held and Kutzner, 1990). The extracellular enzyme appears to be very unstable (Baumann *et al.*, 1976). The absorption spectra of pure tyrosinase shows a maximum at 282nm and a shoulder at 290nm. Another shoulder exists between 320 and 380nm (Lerch and Ettlinger, 1972).

Evidence suggests that the genes for synthesis and secretion of tyrosinase activity are chromosomal (Katz *et al.*, 1983). Intracellular tyrosinase is synthesized *de novo* after the addition of various 2-aminocarboxylic acids to a growing culture. This was shown by Baumann and Kocher in 1974, and excludes the possibility of activation of an inactive precursor of the enzyme. There is no indication of an endogenous inhibitor.

Studies reveal the location of the tyrosinase marker (*tye*) between loci *met-2* and *gua-4* on the chromosome (Figure 4.1), and Baumann and Kocher (1974) suggest an extrachromosomal position for the excretion character (*mel*).

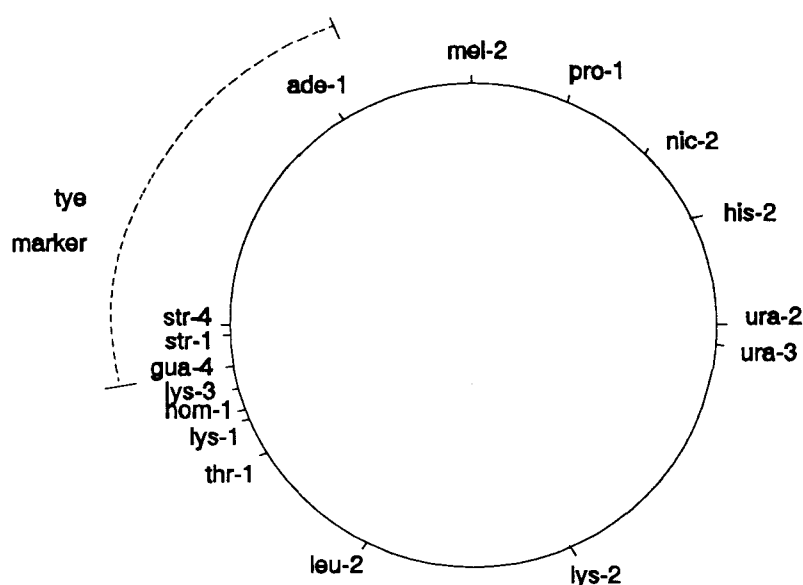


Figure 4.1: Circular linkage map of *S. glaucescens* (Baumann and Kocher, 1974).

The *mel* locus of *S. glaucescens* has a nearly identical ORF sequence upstream of tyrosinase that serves a similar function as in *S. antibioticus* (della-Cioppa *et al.*, 1990).

A complex genetic control of melanin formation seems to exist. Firstly, control by the structural gene for tyrosinase (*tye*) and secondly, by genes controlling the secretion of the enzyme (*mel*). Genes controlling the regulation or induction of the enzyme may also exist as a third group of enzymes (Baumann *et al.*, 1976).

The Streptomycetes are potentially valuable as an alternative source of tyrosinase. They are a newly-discovered but well-known source of the enzyme. Advantages of this source include the formation of a single enzyme and the use of easily available and controllable inducers. It may also be possible to control intracellular versus extracellular enzyme production by *S. glaucescens* to suit the extraction process. *Streptomyces* can also be handled via conventional microbiological techniques (Dworkin, 1985).

Selection of *Streptomyces* cultures

Firstly it is essential to identify and isolate tyrosinase-producing Streptomycetes. These microorganisms can then be manipulated to maximize tyrosinase production; either genetically by mutagenesis, or by alteration of growth conditions under which the enzyme is produced. The latter method was undertaken in this study as growth conditions are more easily controlled for optimum enzyme production.

S. antibioticus, *S. glaucescens* and an engineered strain, *S. lividans* (pIJ702) were selected for study. The latter culture contains a cloned *mel* gene from *S. antibioticus* in *S. lividans* via the plasmid pIJ702. pIJ702 is a broad host-range plasmid vector of copy number 40-300 used exclusively in Streptomycetes. Katz *et al.* (1983) reported on its construction. A *Bcl*I-generated DNA fragment coding for tyrosinase was cloned from *S. antibioticus* 3720 DNA into plasmid vectors to generate hybrid plasmids, using *S. lividans* 66 as host. The 1.55kb fragment was subcloned into the multicopy plasmid pIJ350 to generate pIJ702. This plasmid contains the thiostrepton-resistance determinant (*tsr*) from *S. azureus* and the tyrosinase (*mel*) determinant of *S. antibioticus* (Figure 4.2). It has been suggested that strains of *S. lividans* carrying the genes possess an inducible enzyme activity as in the

S. antibioticus parent. The majority of the enzyme remains intracellular in *S. lividans*. The specific activity of the intracellular induced tyrosinase was up to 36-fold higher in comparison to low-copy plasmids, and *S. antibioticus*.

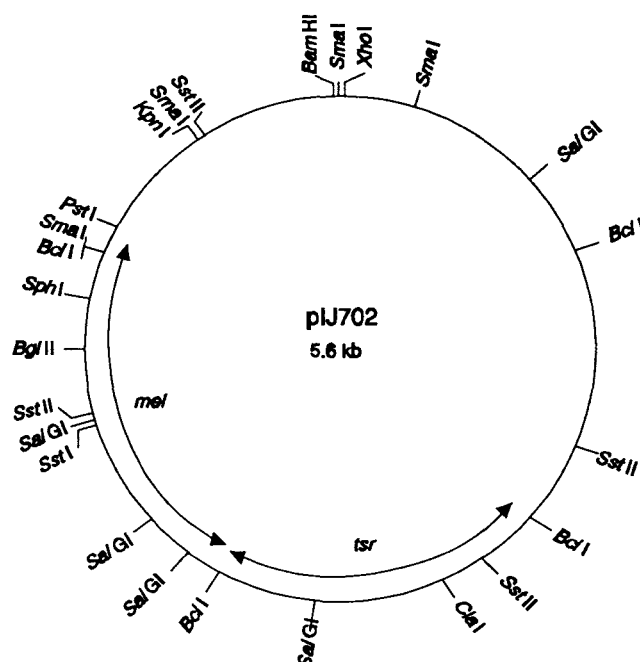


Figure 4.2: Restriction map of pIJ702 (Katz *et al.*, 1983).

The application of genetic engineering for enhancing enzyme production has much potential for the production of novel products. Reactions other than those catalyzed by naturally occurring enzymes can therefore be specifically carried out at rates comparable to natural enzymes and under similarly mild conditions. However, the expression of the introduced genetic material is often uncertain and variable.

The various stages by which the selected Streptomyces were screened for tyrosinase production are outlined below, together with references to the methods used.

1. Passaging microorganisms onto solid substrate - section 2.2, appendix 2.
2. Enzyme production in batch culture systems - section 2.3.2, appendix 4.
3. Enzyme induction studies - section 2.4.2.

4. Enzyme extraction - section 2.8.
5. Enzyme assays - sections 2.11.1 and 2.11.4.
6. Protein determinations and zymogram analysis - sections 2.12 and 2.13, appendices 6 and 7.
7. Partial purification of enzyme extracts by salt fractionation - section 2.14, appendix 8.
8. Enzyme catalysis in organic media and product analysis - sections 2.19 and 2.20.3.3.

4.2. RESULTS

4.2.1. Organisms on solid substrate (plate tests)

Tyrosinase production by selected cultures was confirmed by the presence of black eumelanin surrounding colonies on tyrosine-containing agar plates (Chapter 3, plates 3.1 - 3.3). This colouration reaction demonstrates the enzymatic oxidation of tyrosine to melanin. A non tyrosinase-producing streptomycete, *S. griseus*, was used as control. No colour change was observed.

4.2.2. Enzyme production in shaker-flask cultures

All shaker-flask experiments were carried out in duplicate and results expressed as a mean value.

4.2.2.1. Enzyme production in M3 medium

Spore-inoculated (S1) and the first set of broth-inoculated flasks (B1) showed distinct colour changes from golden to brown during incubation, with *S. antibioticus* being the darkest of the S1 flasks. Due to the heavy growth in B1 flasks, cultures (10%) were passaged into B2 shaker flasks. By the end of the 7 day culture period B2 flasks also showed confluent growth, but no colour changes. Samples from B2 flasks were streaked onto tyrosine-containing M3 agar plates to confirm enzyme presence. Results were positive, indicating that tyrosinase was still catalytically active.

After extraction, B1, B2 and S1 samples were assayed for tyrosinase against phenol and tyrosine and results expressed as units of enzyme activity.ml⁻¹.

Table 4.1: Results of *Streptomyces* shaker-flask cultures B1 and B2 tyrosinase assayed against phenol and tyrosine (units of activity.ml⁻¹).

CULTURE	BROTH INOCULATED 1				BROTH INOCULATED 2			
	Intracellular		Extracellular		Intracellular		Extracellular	
	p	t	p	t	p	t	p	t
<i>S.antibioticus</i>	1 669	385	1 854	592	4 177	5 500	839	700
<i>S.glaucescens</i>	2 215	2 300	715	1 815	92	3 915	1 292	2 477
<i>S.lividans</i> (pIJ702)	2 723	5 508	1 638	692	1 054	4 554	-	-

Assay substrates: t - tyrosine
p - phenol

Table 4.2: Results of *Streptomyces* shaker-flask cultures S1 tyrosinase assayed against phenol and tyrosine (units of activity.ml⁻¹).

CULTURE	SPORE INOCULATED 1			
	Intracellular		Extracellular	
	p	t	p	t
<i>S.antibioticus</i>	2 746	6 000	2 215	1 877
<i>S.glaucescens</i>	2 123	254	592	908
<i>S.lividans</i> (pIJ702)	1 808	1 485	-	-

Assay substrates: t - tyrosine
p - phenol

These results indicate predominantly intracellular enzyme production by *S. lividans* (pIJ702) and both intra- and extracellular enzyme production by *S. glaucescens* and *S. antibioticus*. Activities against phenol and tyrosine were quite different and little correlation was seen between these results. Passaging appeared to have little meaningful effect on enzyme production, but did enhance tyrosine:phenol activity ratios, possibly due to improved uptake of tyrosine.

Two extracts were selected on the basis of good activity for modified aeration experiments in an organic solvent. These experiments were carried out in chloroform so as to retard the reaction in an attempt to identify reaction intermediates and elucidate the tyrosinase reaction sequence. Samples were analyzed by capillary-GC using a SE 30 column (section 2.20.3.3).

1. *S. antibioticus* B2 (intracellular) extract - aqueous and freeze-dried (concentrated) samples: Figures 4.3 - 4.6 compare the phenol removal ability of *A. bisporus* crude tyrosinase dried extract and *S. antibioticus* B2 samples of similar enzyme strength. The diluting effect of the aqueous *S. antibioticus* sample was seen in the reduced phenol starting concentration. No reaction intermediates were detected.

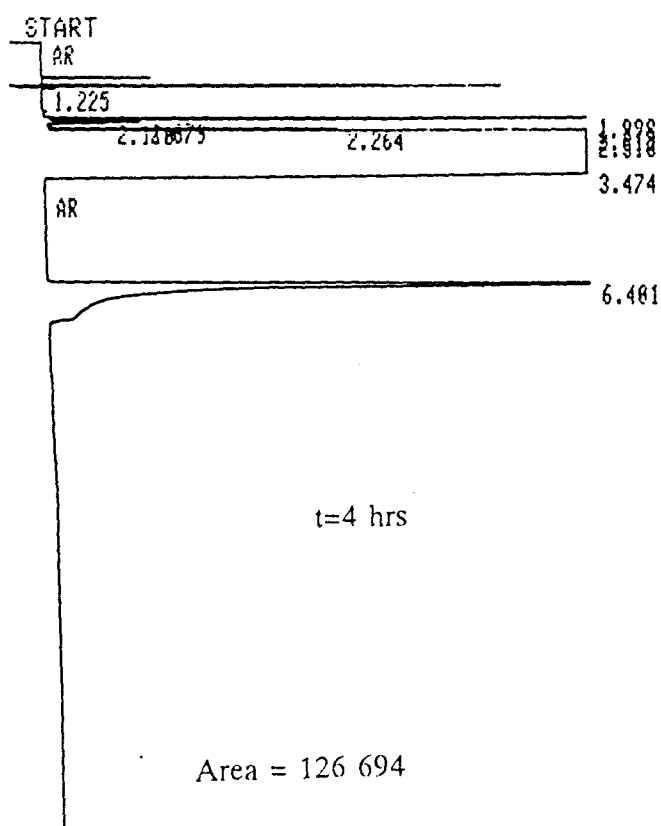
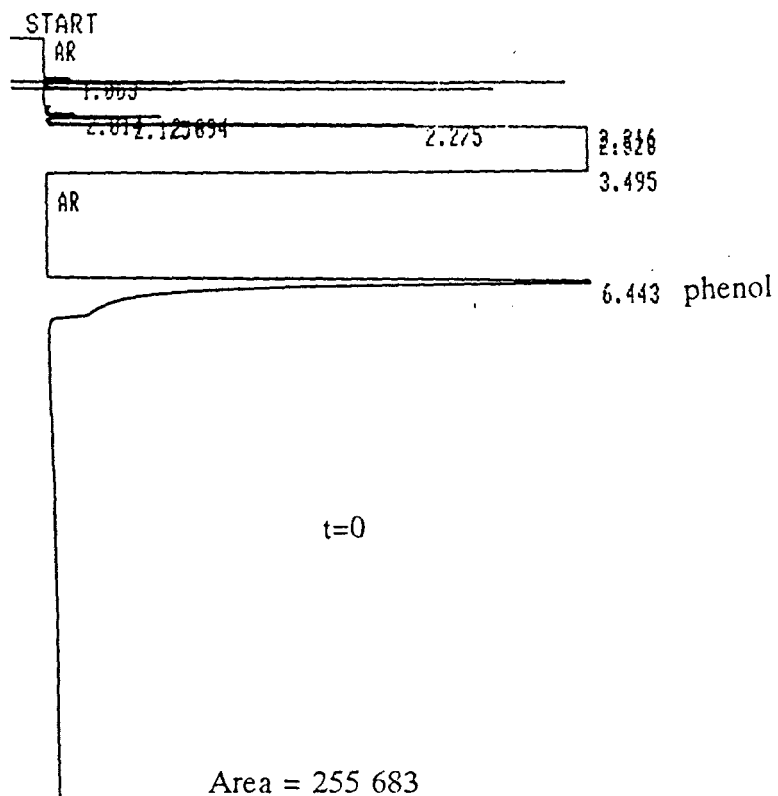


Figure 4.3: Phenol removal efficiency of *A. bisporus* crude tyrosinase (dried extract) at 0 and 4 hours.

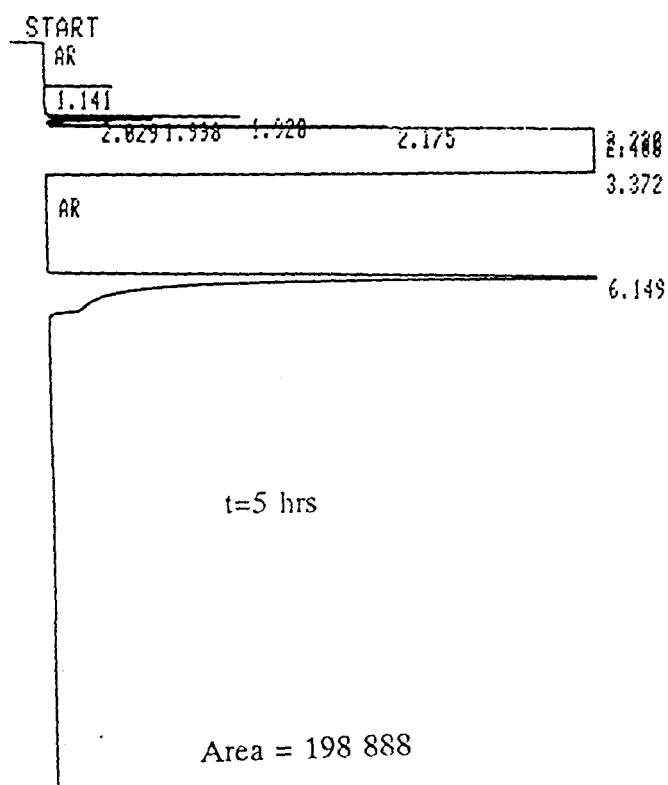
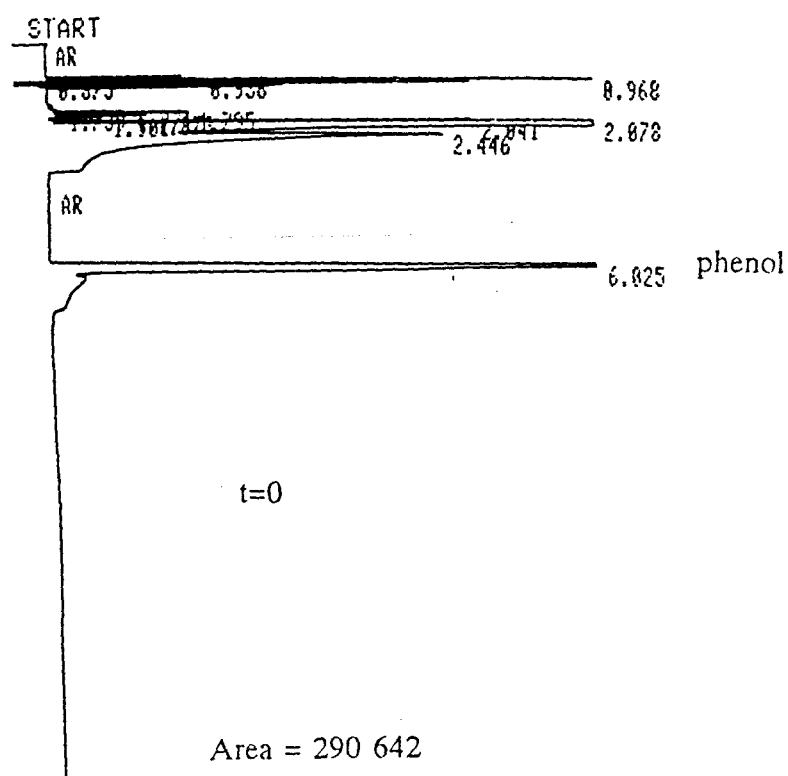


Figure 4.4: Phenol removal efficiency of *S. antibioticus* (B2 intracellular aqueous extract) at 0 and 5 hours.

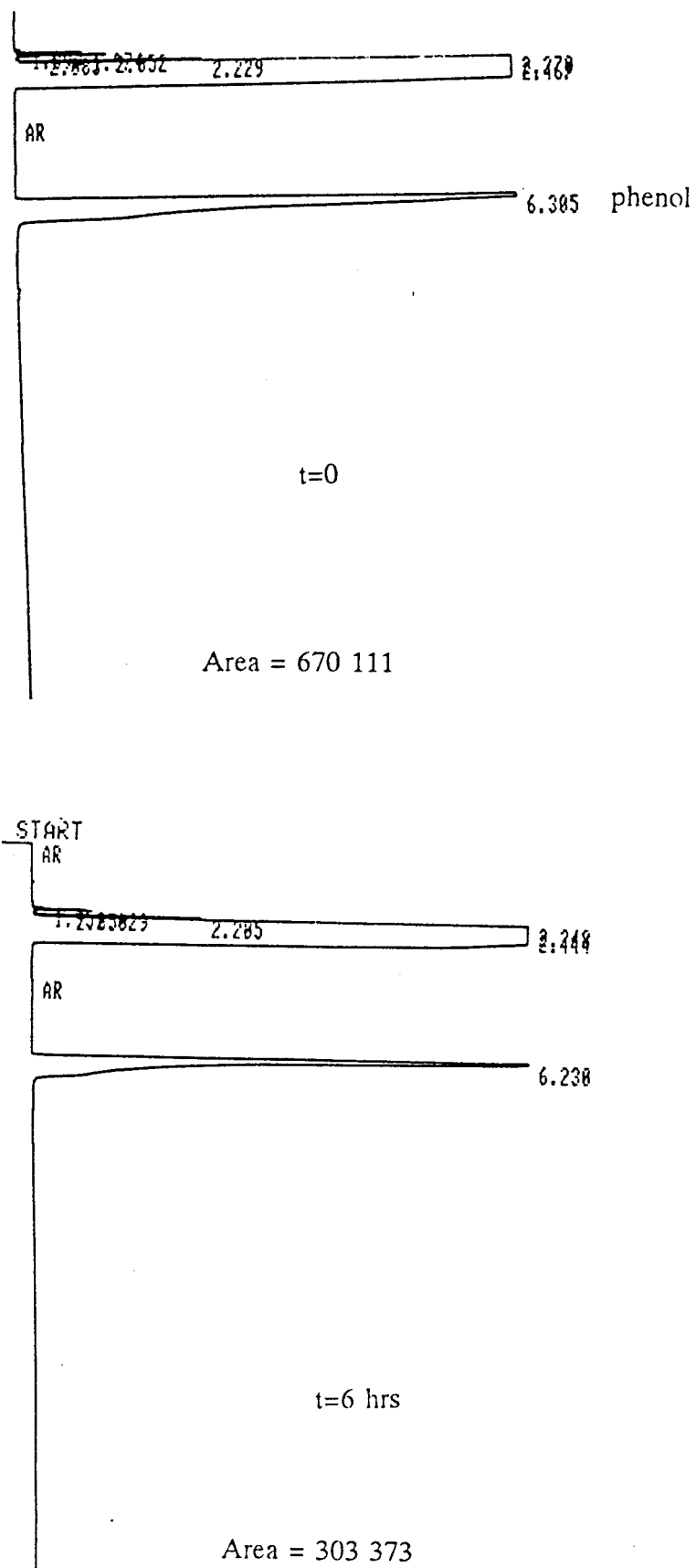


Figure 4.5: Phenol removal efficiency of *S. antibioticus* (B2 intracellular dried extract) at 0 and 6 hours.

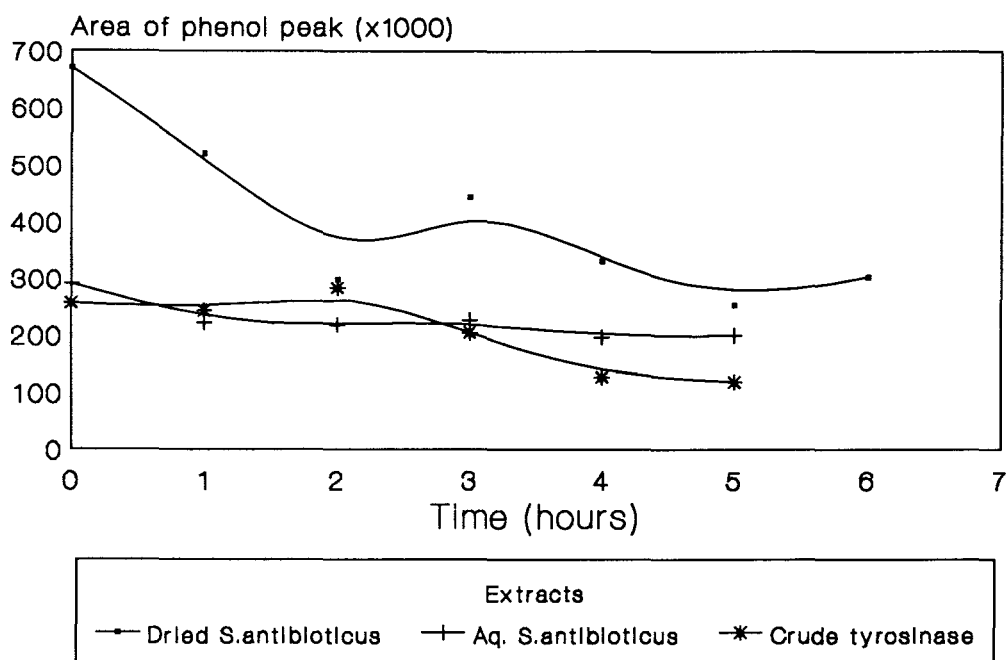


Figure 4.6: A comparison of phenol removal efficiencies of *S. antibioticus* extracts and *A. bisporus* crude tyrosinase.

2. *S. lividans* (pIJ702) B2 (intracellular) extract - concentrated sample: Figure 4.7 shows the reduction in phenol catalyzed by *S. lividans* (pIJ702) as monitored by GC, and figure 4.8 compares the removal efficiencies of *A. bisporus* aqueous enzyme extract and *S. antibioticus* and *S. lividans* (pIJ702) freeze-dried samples. The diluting effect of the aqueous sample on starting concentrations is again evident. Again, no identifiable intermediates were detected. An unidentifiable peak was detected at 3.7 minutes after 5 hours of the *S. lividans* (pIJ702) experiment.

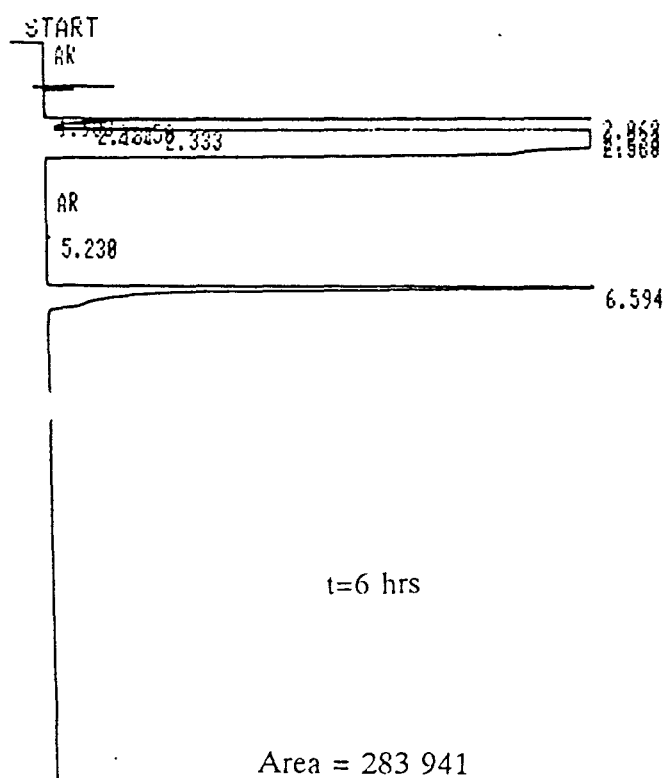
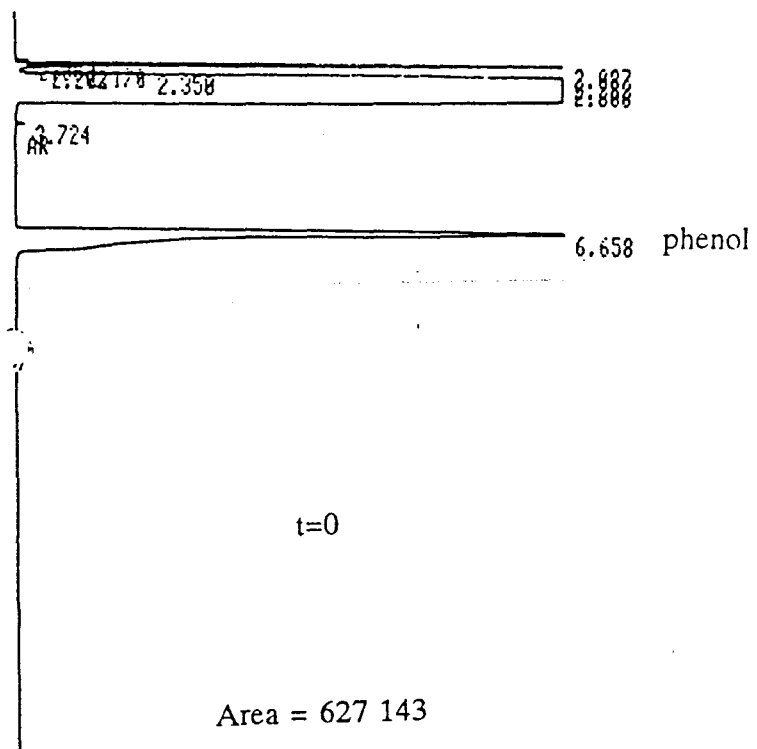


Figure 4.7: Phenol removal efficiency of *S. lividans* (pIJ702) (B2 intracellular dried extract) at 0 and 6 hours.

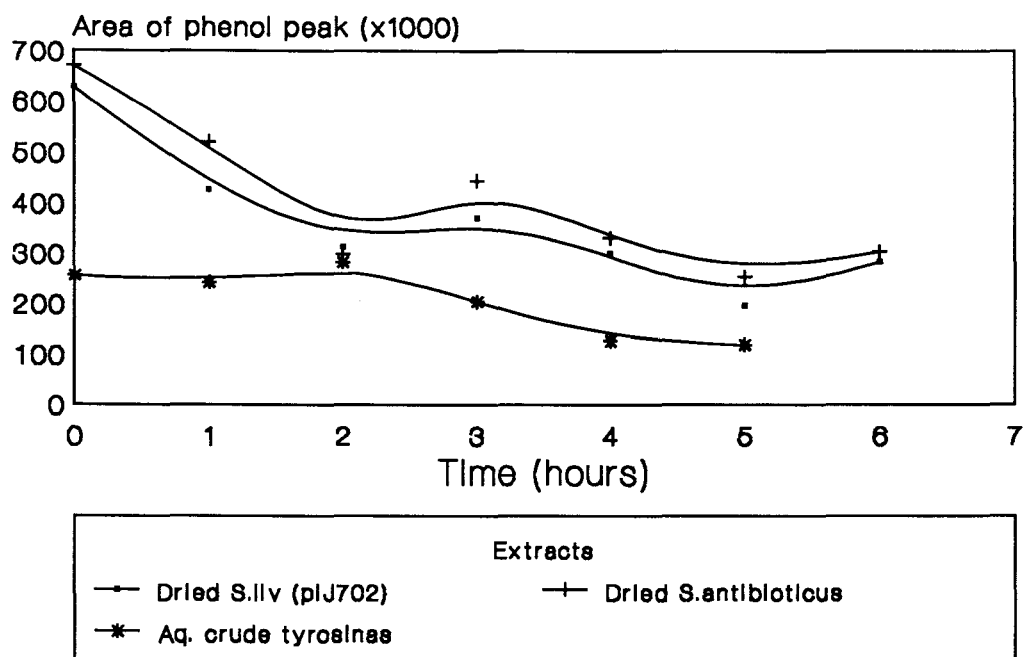


Figure 4.8: A comparison of phenol removal efficiencies of *S. antibioticus*, *S. lividans* (pIJ702) and *A. bisporus* crude tyrosinase.

Concentrated (freeze-dried) samples showed phenol removal, but the reaction sequence could not be elucidated as intermediates were not detected. All enzyme extracts were reassayed two months later, but no enzyme activity was found. This result suggests the instability of the Streptomycete enzyme.

4.2.2.2. Enzyme production in GYM medium - Experiment a

A slight darkening of growth media was observed over the enzyme-producing period; the darkest culture after 35 hours being *S. antibioticus*, pH 7.2. A marked difference was apparent in biomass production at pH 7.2 and pH 9 (Figure 4.9) and also *S. antibioticus* and *S. glaucescens* pH 7.2 flasks (Figure 4.10). Biomass production was low.

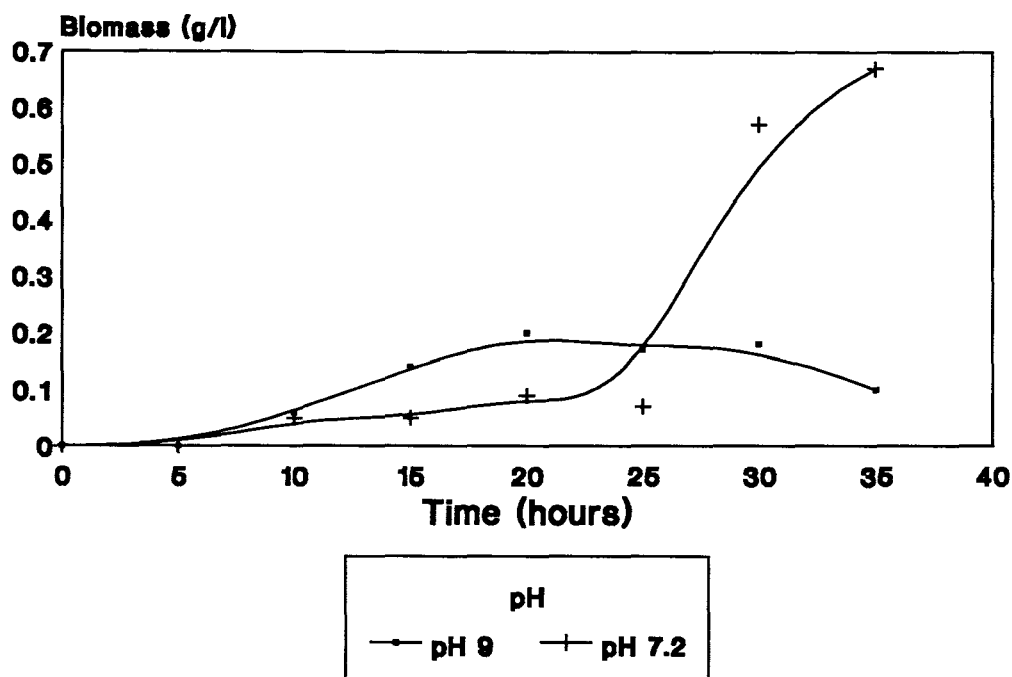


Figure 4.9: A comparison of biomass production by *S. antibioticus* at pH 7.2 and pH 9.

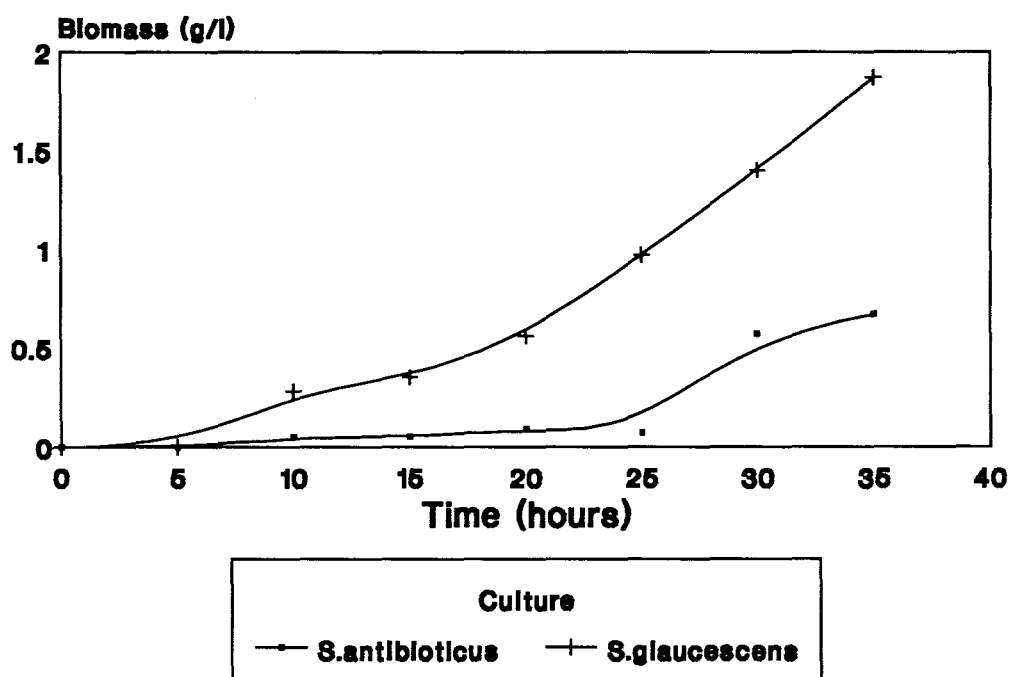


Figure 4.10: A comparison of biomass production by *S. antibioticus* and *S. glaucescens* in pH 7.2 GYM media.

As Streptomycete tyrosinase shows a 30-fold increase of activity on L-DOPA vs. L-tyrosine, and a 60-fold increase over phenol (Philipp *et al.*, 1991), L-DOPA was selected as enzyme substrate for subsequent induction studies. This also enables a comparison of results with published values. All extracts were therefore assayed against L-DOPA and activities calculated as units of enzyme activity.ml⁻¹ using the Beer-Lambert Law i.e. $A=\epsilon cl$, with ϵ for dopachrome = 3 600 (Lerch and Ettlinger, 1972). Results are shown in table 4.3. No intracellular activity was found and no activity was detected in pH 9 cultures. These findings clearly demonstrate the determining role of growth medium and pH in enzyme production.

Table 4.3: Extracellular tyrosinase production of *Streptomyces* cultures grown in pH 7.2 GYM media expressed as units of enzyme activity.ml⁻¹.

TIME (hrs)	<i>S. glaucescens</i>	<i>S. antibioticus</i>
0	0	0
10	0	0
15	0	0
20	33.3	0
25	38.8	0
30	40.2	41.7
35	13.2	63.8

Figures 4.11 and 4.12 demonstrate the relationship between glucose utilization, biomass production and enzyme activity in pH 7.2 GYM media for *S. antibioticus* and *S. glaucescens* respectively. Enzyme activity is closely linked to biomass production, while biomass and glucose utilization are inversely related. *S. antibioticus* is the better enzyme producer per gram biomass.

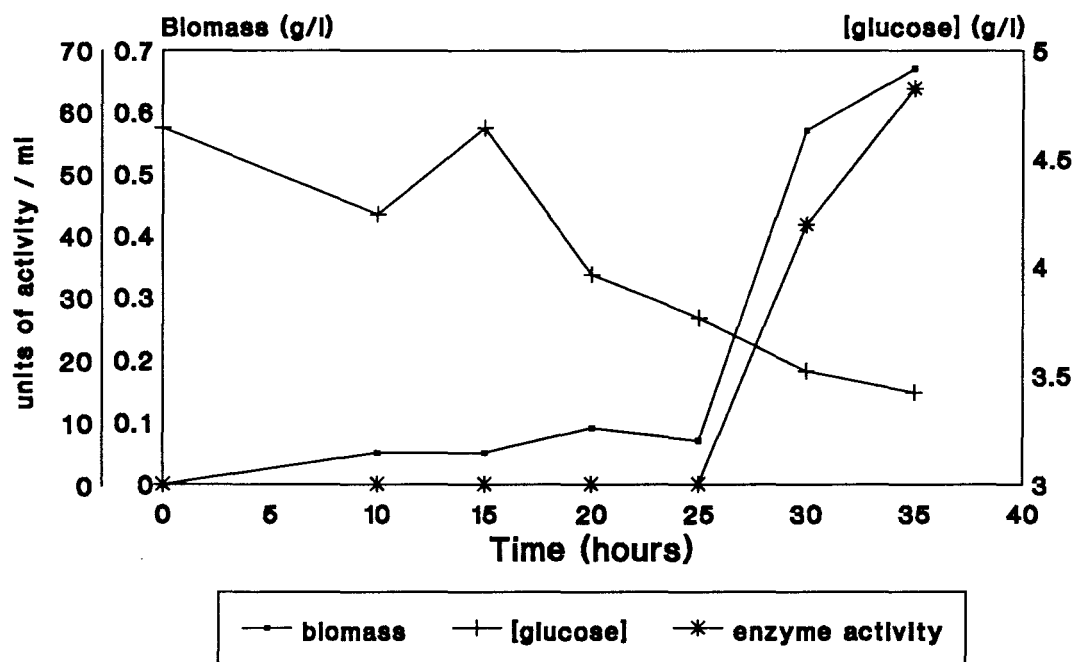


Figure 4.11: *S. antibioticus* in pH 7.2 GYM medium.

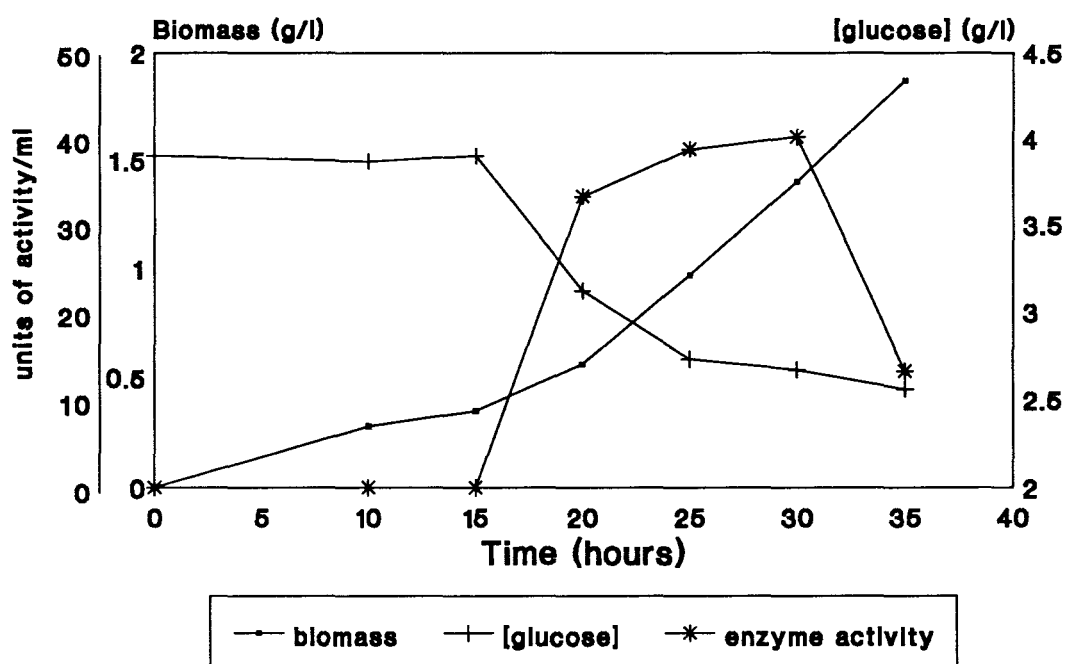


Figure 4.12: *S. glaucescens* in pH 7.2 GYM medium.

Control experiment flasks were inoculated with *S. antibioticus* spore suspensions and treated as before. The results were as follows:

Control 1: Maintaining culture temperature at 30°C resulted in high biomass production i.e. approximately 2 g.l⁻¹, but lower extracellular enzyme production i.e. 14 units.ml⁻¹. Maximum enzyme production was at 34 hours.

Control 2: No aeration resulted in minimal biomass production and therefore no enzyme production.

Control 3: As original glucose was still available to the cells at the end of the experiment, the addition of extra carbon and nitrogen sources did not notably affect enzyme production.

Control 4: The addition of methionine to culture media did not notably increase enzyme production.

The protein content of enzyme-containing fractions was determined by Bradford's method (Table 4.4) in preparation for zymogram analysis - see plate 4.1.

Table 4.4: Protein determinations of extracted samples.

ENZYME EXTRACTS	PROTEIN CONCENTRATION (mg.ml ⁻¹)
<i>S. glaucescens</i> (20 hours)	0.65
<i>S. glaucescens</i> (25 hours)	0.72
<i>S. antibioticus</i> (30 hours)	0.91
<i>S. antibioticus</i> (35 hours)	0.50

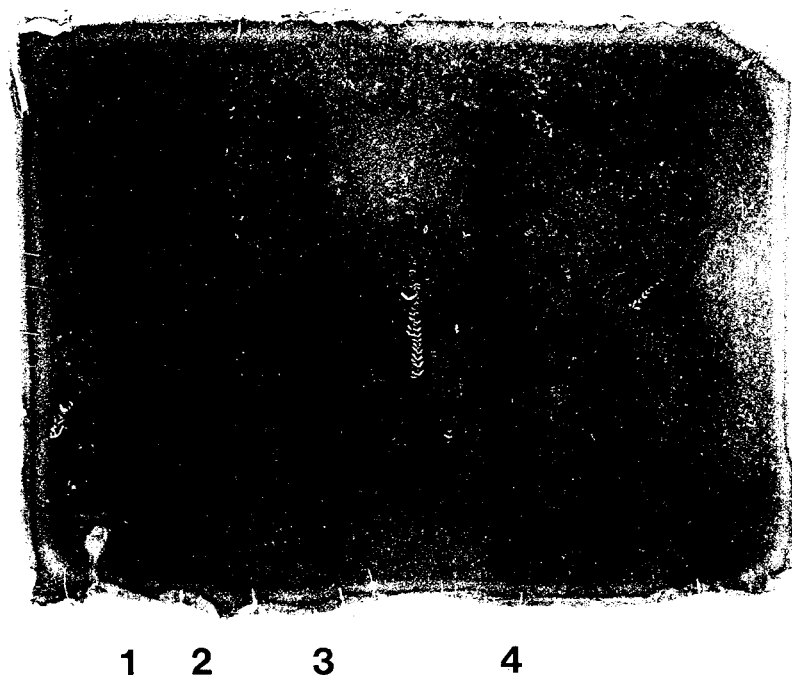


Plate 4.1: Zymogram developed against L-DOPA.

Lane 1: *S. glaucescens*, extracellular enzyme, 20 hours.
Lane 2: *S. glaucescens*, extracellular enzyme, 25 hours.
Lane 3: *S. antibioticus*, extracellular enzyme, 30 hours.
Lane 4: *S. antibioticus*, extracellular enzyme, 35 hours.

Plate 4.1 serves merely as an indicator of enzyme presence. No distinct enzyme spots were seen, but melanin streaks were apparent in lanes 1-4 on photographing. Attempts to improve zymogram resolution were unsuccessful. The reassay of samples two months later showed no enzyme activity. A second zymogram confirmed this result, confirming enzyme instability.

Experiment b

Due to low biomass formation in experiment a, the experiment was repeated with the 30°C biomass production period being extended to 20 hours. A 10% increase in biomass was achieved. Excellent enzyme production was recorded on assay against L-DOPA, and results are shown in table 4.5. *S. glaucescens* again grew more rapidly than *S. antibioticus*.

The relationship between total enzyme activity and biomass is presented in figures 4.13 and 4.14.

Table 4.5: *S. antibioticus* and *S. glaucescens* tyrosinase production in pH 7.2 GYM media calculated as units of enzyme activity.ml⁻¹.

TIME (hrs)	<i>S.glaucescens</i>		<i>S.antibioticus</i>	
	Extracellular	Intracellular	Extracellular	Intracellular
0	0	0	0	0
20	477.7	263.8	43.2	19.2
28	377.7	187.5	120.9	107.2
36	338.1	78.6	225.3	157.5
44	446.4	93.3	500.6	184.7
52	673.1	109.4	477.7	67.3

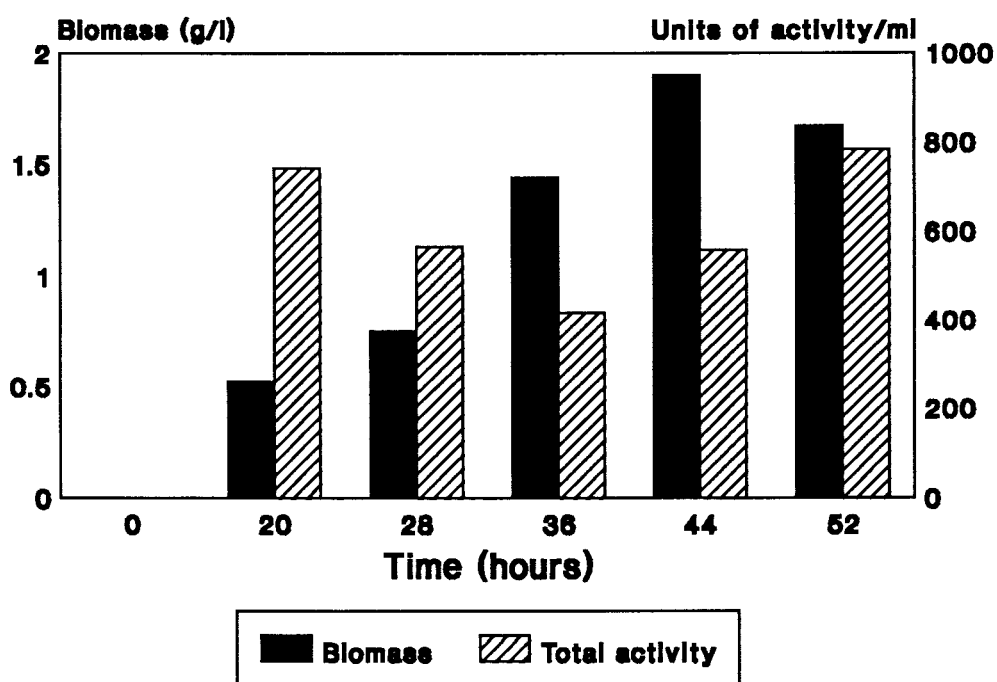


Figure 4.13: The relationship between enzyme activity and biomass production for *S. glaucescens* in pH 7.2 GYM medium.

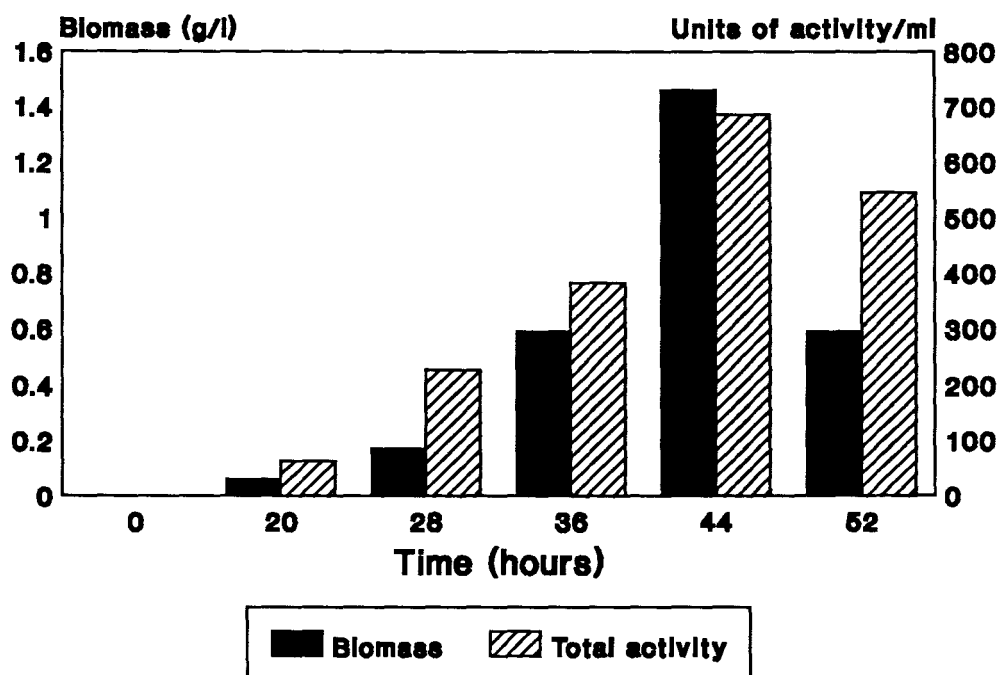


Figure 4.14: The relationship between enzyme activity and biomass production for *S. antibioticus* in pH 7.2 GYM medium.

As in the previous experiment, the inclusion of methionine into culture media did not notably increase enzyme production.

Enzyme assays were repeated 4 months later and, although still meaningful as compared to results achieved during other screening procedures, activities had dropped between 70% and 80%. The *Streptomyces* tyrosinase may therefore be viable for immediate use upon extraction, but not for long-term storage.

4.2.2.3. Enzyme production in GYM medium using *S. lividans* (pIJ702)

Biomass production was monitored visually throughout the reaction (Table 4.6).

Table 4.6: Biomass production by *S. lividans* (pIJ702) in pH 7.2 GYM medium.

EXPERIMENT	BIOMASS PRODUCTION
Experiment 1	+
Experiment 2	+++
Experiment 3	++++
Experiment 4	++

+: little biomass production
++: good biomass production
+++: very good biomass production
++++: excellent biomass production

Experiment 1: A two-temperature strategy was maintained as in section 2.4.2.2.
Experiment 2: The 30°C period allowed for biomass production was doubled to 20 hours and the experiment continued at 25°C for another 52 hours.
Experiment 3: Culture temperature was maintained at 30°C.
Experiment 4: Culture temperature was maintained at 25°C.

See figure 4.15 for a graphical presentation of biomass production recorded at the end of the experiment. Growth at 30°C stimulated biomass production, but samples assayed showed no notable enzyme activity.

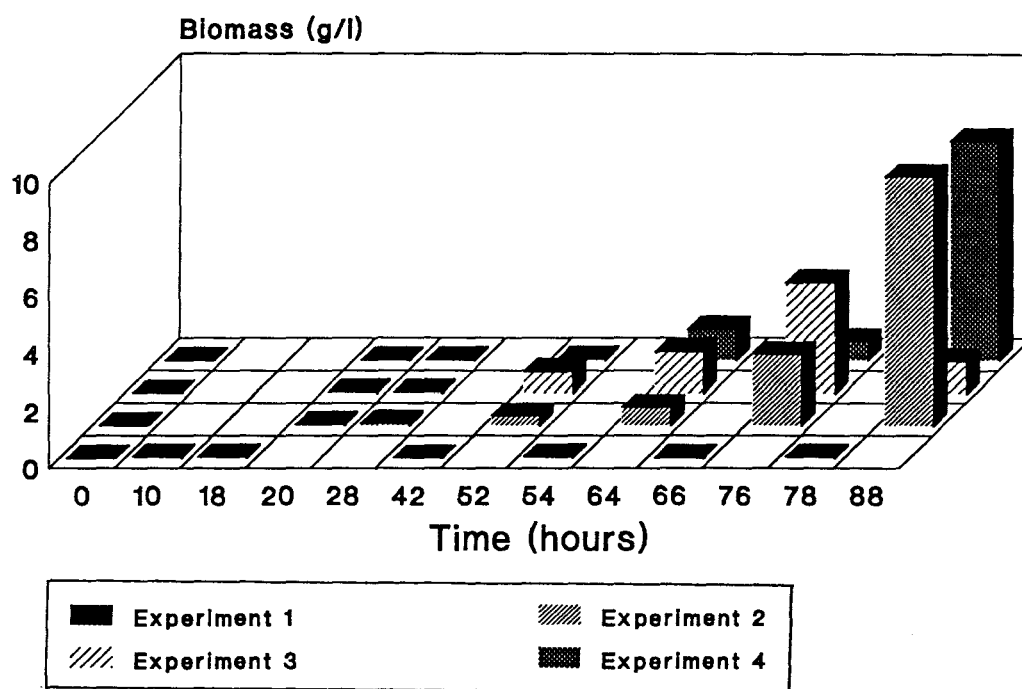


Figure 4.15: Biomass production by *S. lividans* (pIJ702) in pH 7.2 GYM medium.

4.2.2.4. Enzyme production by *S. lividans* (pIJ702) in various media

Biomass production monitored throughout the experiments was minimal. MMT was the only media which showed any colour change (Plate 4.2). The change in colour started after 24 hours and by day 7 media was black with a black precipitate. Extracellular fractions showed a distinct black meniscus. The production of black eumelanin by the action of tyrosinase on tyrosine was therefore clearly shown in MMT media.

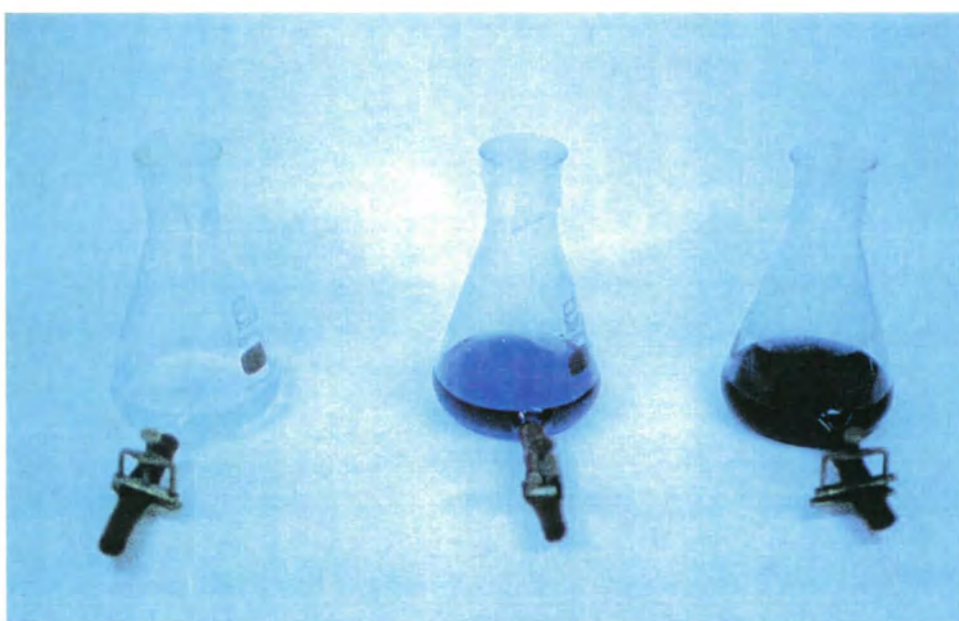


Plate 4.2: A change of MMT medium colour from clear to dark blue on inoculation with *S. lividans* (pIJ702) colonies.

Only MMT intracellular fractions showed enzyme activity. A peak of 27 units of activity.ml⁻¹ after 5 days, and a drop back to 10 units after 7 days (Figure 4.16) was observed. These assays were repeated one week later, but no enzyme was detected, again suggesting enzyme instability. To confirm the loss of enzyme shown on assay, the protein content of the MMT intracellular extract was determined (0.4 mg.ml⁻¹) and the sample run on a zymogram (Plate 4.3). Aqueous and dried crude *A. bisporus* tyrosinase extracts were run as standards (see table 4.10 for R_f values). Contrary to assay results, melanin "spots" were clearly visible on the gel, indicating the presence of catalytically active tyrosinase. This result demonstrates the greater sensitivity of zymograms over enzyme assays for the detection of low enzyme concentrations.

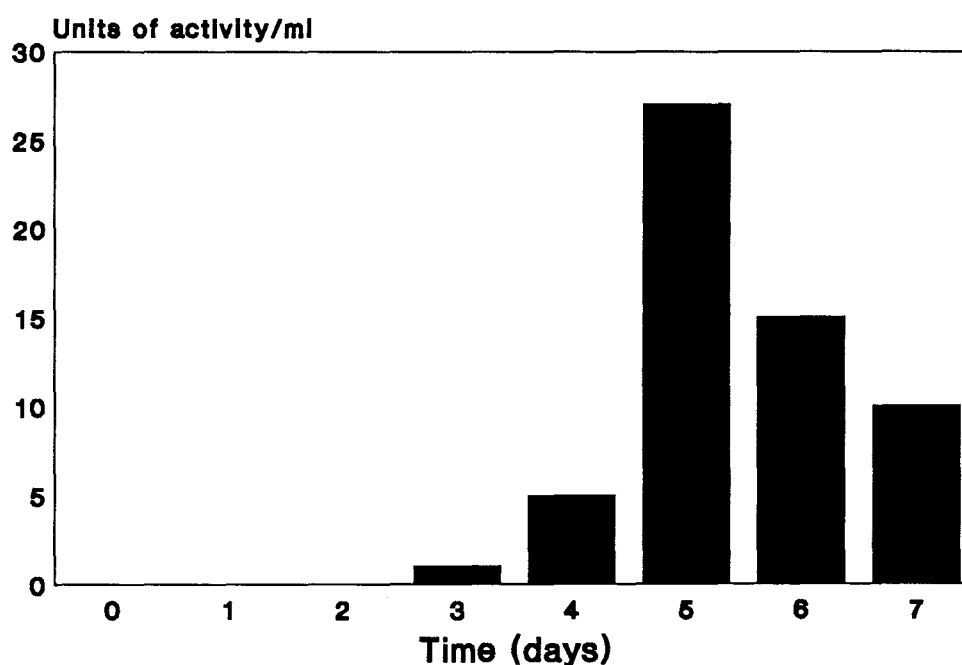


Figure 4.16: Tyrosinase production by *S. lividans* (pIJ702) in pH 8 MMT medium.



Plate 4.3: Zymogram developed against L-DOPA.

Lane 1: *S. lividans* (pIJ702) pH 8, MMT-induced tyrosinase.
Lane 2: Crude aqueous *A. bisporus* tyrosinase.
Lane 3: Crude dried *A. bisporus* tyrosinase.

Plate 4.3 was the first indication of enzyme multiplicity in the Streptomyces. Although *Streptomyces* tyrosinase is known to be a single enzyme due to the existence of a single polypeptide chain (Bernan *et al.*, 1985; Huber *et al.*, 1985; Katz and Bétancourt, 1988), two enzyme bands were seen on zymogram analysis. Two forms of the enzyme may therefore be produced under these reaction conditions. This phenomenon may be caused by pH-induced changes to the tertiary structure of the enzyme.

Intra- and extracellular enzyme fractions were analyzed on capillary-GC (SE 30 column) in an attempt to detect reaction intermediates. Standard elution times were as follows:

p-quinone:	5.51 minutes
phenol:	6.63 minutes
p-cresol:	9.23 minutes
catechol:	12.38 minutes

As the extracellular extract had a prominent black precipitate, two samples were analyzed. One after shaking (Figure 4.17a), and another from the clear zone which developed upon settling of the sample (Figure 4.17b). Results show that upon removal of the precipitate only one oxidation product remains, peaking at 3.9 minutes. Despite an attempt to analyze the sample using gas chromatography-mass spectroscopy (GC-MS), it was not possible to identify this intermediate. No peaks were seen upon analysis of the intracellular extract.

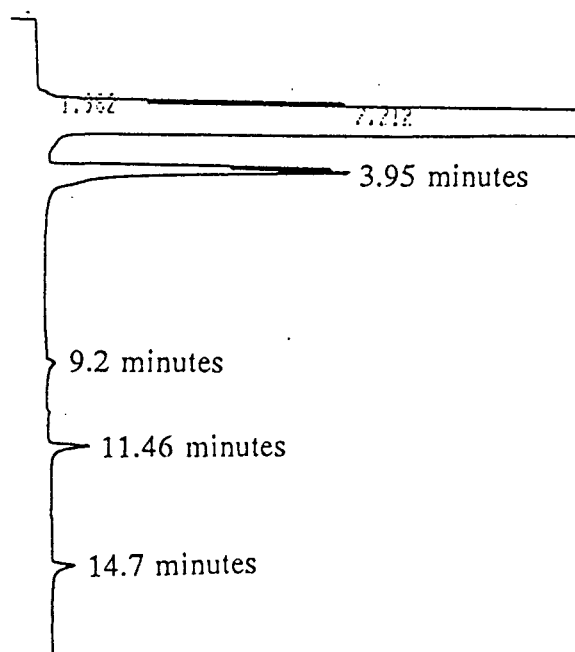


Figure 4.17a: Extracellular extract of *S. lividans* (pIJ702) in pH 8 MMT medium (homogeneous sample).

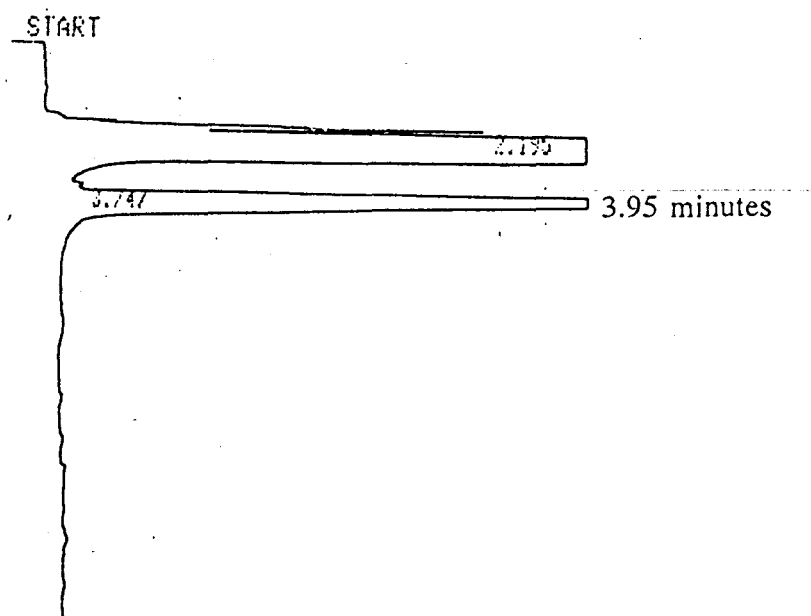


Figure 4.17b: Extracellular extract of *S. lividans* (pIJ702) in pH 8 MMT medium (extract from clear zone of settled sample).

The MMT intracellular sample was centrifuged to remove the precipitate and an ammonium sulphate purification was performed (method in appendix 8). This sample was analyzed on a zymogram to determine the effect of purification on enzyme activity. No enzyme spots were seen, suggesting the enzyme was contained within the precipitate, or lost during purification.

4.2.2.5. The effect of pH on *S. lividans* (pIJ702) in MMT

As in experiment 2.4.2.4, an intense colour change was seen. Biomass production and pellet formation was monitored and described in table 4.7.

Table 4.7: Biomass production of *S. lividans* (pIJ702) cultures at different pHs.

pH	BIOMASS (g.l ⁻¹)	DESCRIPTION OF BIOMASS
pH 7.2	0.036	Small pellet and not cohesive.
pH 7.4	0.390	Very large and cohesive.
pH 7.6	0.046	Fairly large; not cohesive. Media very dark and granular. A high degree of polymerization.
pH 8.0	0.014	Very small pellet. Media very dark and granular. A fairly high degree of polymerization.

Attempts at assaying pH 7.4 impure enzyme samples and determining protein concentration were unsuccessful due to the presence of a thick black precipitate which could not be clarified. Tyrosinase was therefore only detected in intracellular fractions (Figure 4.18). The red-brown colouration typical of tyrosine oxidation by tyrosinase was clearly seen on assay of the pH 7.6 extract.

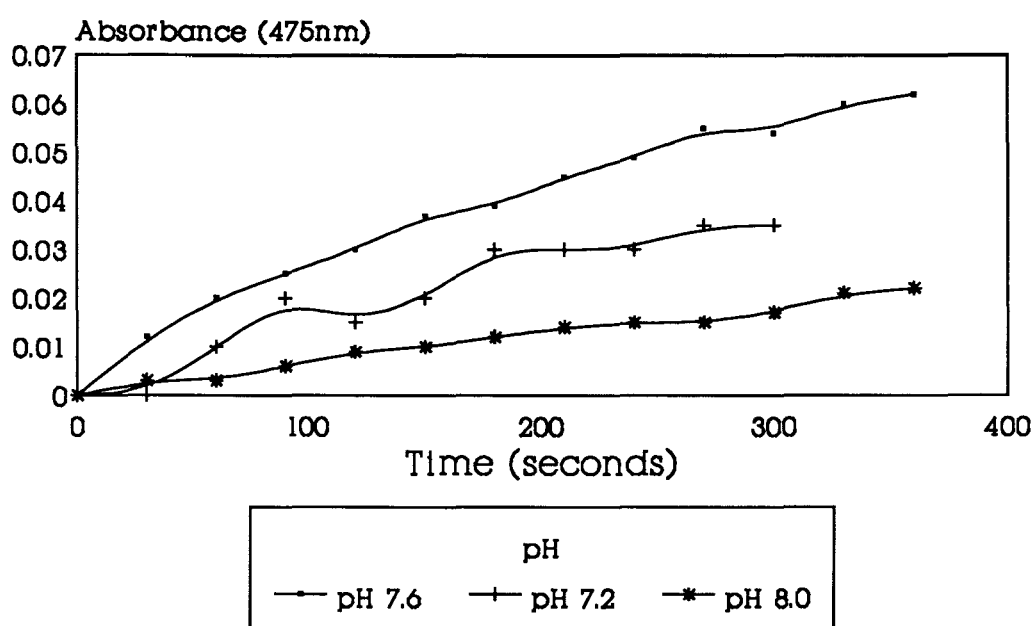


Figure 4.18: Enzyme activities of *S. lividans* (pIJ702) extracts grown in MMT medium at various pH values and assayed against L-DOPA.

In an attempt to prevent enzyme-precipitate binding, ammonium sulphate purification was carried out immediately after extraction and samples assayed at each stage of the purification process (Table 4.8) - see figure 4.19 for assays of pH 7.6 samples.

Table 4.8: *S. lividans* (pIJ702) tyrosinase activities assayed against L-DOPA (units of activity.ml⁻¹) after ammonium sulphate purification.

SAMPLE	ACTIVITY AT DIFFERENT pHs			
	pH 7.2	pH 7.4	pH 7.6	pH 8.0
impure	20.2	unable to assay	28.0	9.7
40% spnt.	minimal	0	minimal	0
60% spnt.	0	0	minimal	0
60% pellet	0	0	22.0	0
70% spnt.	0	0	0	0
pure	minimal	0	0	0

spnt.: supernatant
40% spnt.: supernatant collected after the addition of ammonium sulphate (40% saturation), precipitation of protein on ice and subsequent centrifugation.
60% spnt.: supernatant collected after the addition of ammonium sulphate (60% saturation), precipitation of protein on ice and subsequent centrifugation.
60% pellet: pellet collected after the addition of ammonium sulphate (60% saturation), precipitation of protein on ice and subsequent centrifugation.
70% spnt.: supernatant collected after the addition of ammonium sulphate (70% saturation), precipitation of protein on ice and subsequent centrifugation.
pure: pellet collected after the addition of ammonium sulphate (70% saturation), precipitation of protein on ice and subsequent centrifugation.

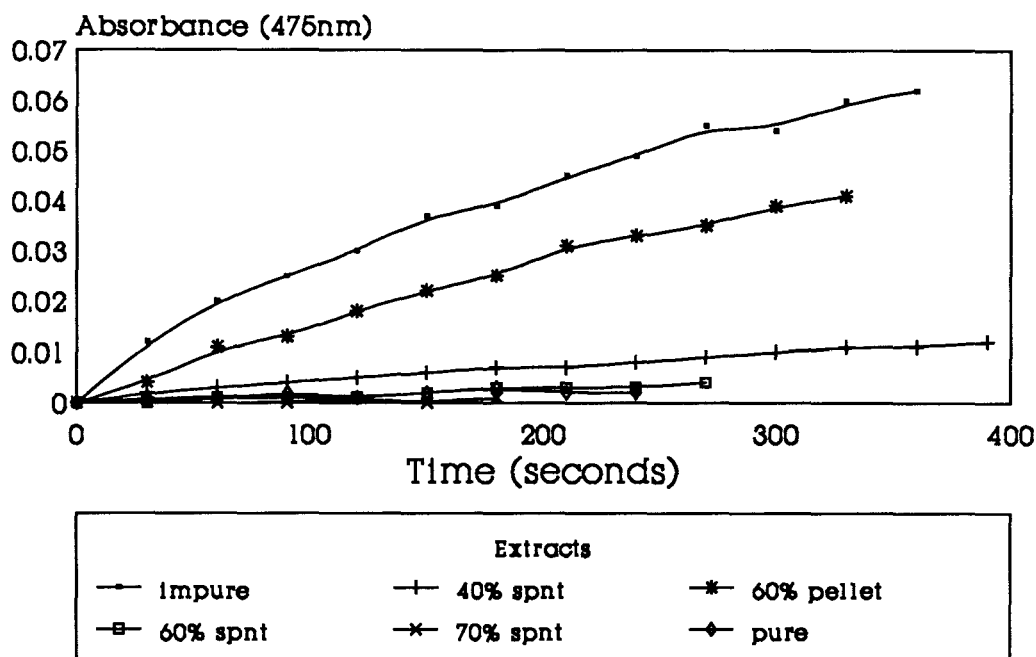


Figure 4.19: Enzyme activities of *S. lividans* (pIJ702) pH 7.6 extracts assayed against L-DOPA after salt purification.

Folin-Lowry determinations on pH 7.2 pure and impure samples showed protein concentrations of 0.22 and 2.2 mg.ml⁻¹ respectively and 0.4 mg.ml⁻¹ for the pH 8 sample. As insufficient material was available, protein determinations could not be carried out on pH 7.6 extracts. Pure and impure pH 7.2 and all pH 7.6 samples were run on zymograms (see table 4.10 for R_f values) using 40μl loading volumes. A crude *A. bisporus* tyrosinase extract was run as a standard, shown in plate 4.4. This zymogram clearly shows enzyme activity in pH 7.2 samples. It was repeated some weeks later to determine enzyme stability (Plate 4.5). Aqueous and dried crude *A. bisporus* tyrosinase, as well as commercial tyrosinase extracts were run as standards. Plate 4.5 clearly shows enzyme spots, proving the stability of *S. lividans* (pIJ702) tyrosinase produced at this pH. Plate 4.6 shows the activity of pH 7.6 fractions. Crude *A. bisporus* tyrosinase was run as positive standard. A comparison of R_f values are represented in table 4.10.

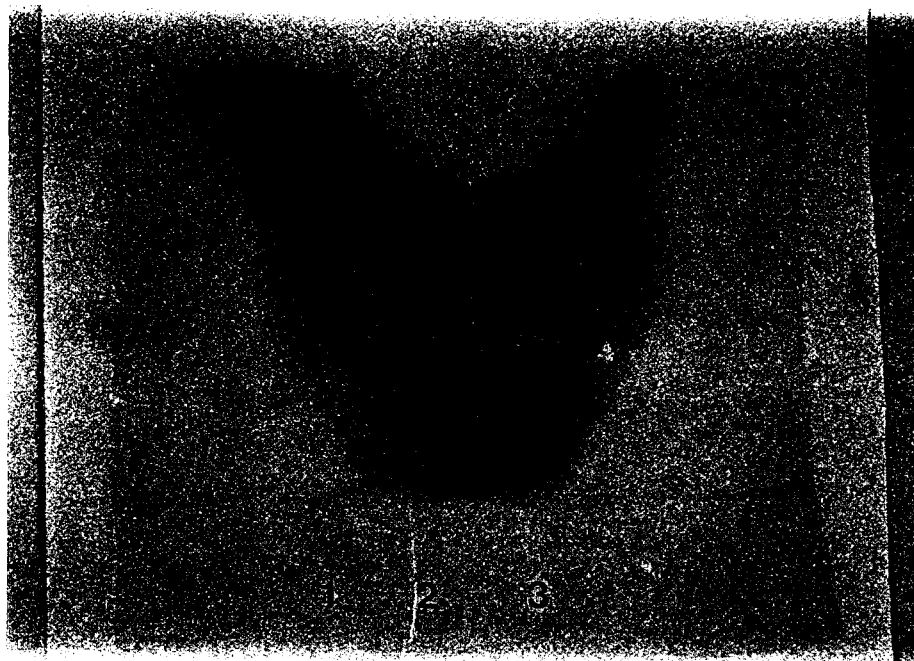


Plate 4.4: Zymogram developed against L-DOPA.

Lane 1: *S. lividans* (pIJ702) pH 7.2, MMT-induced pure tyrosinase.

Lane 2: Crude aqueous *A. bisporus* tyrosinase.

Lane 3: *S. lividans* (pIJ702) pH 7.2, MMT-induced impure tyrosinase.

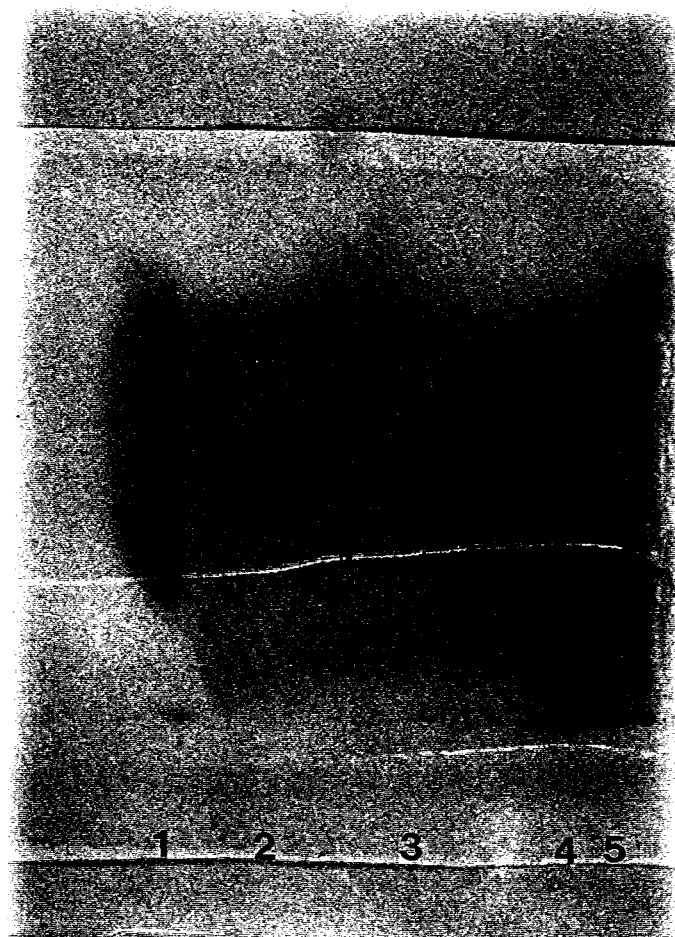


Plate 4.5: Zymogram developed against L-DOPA.

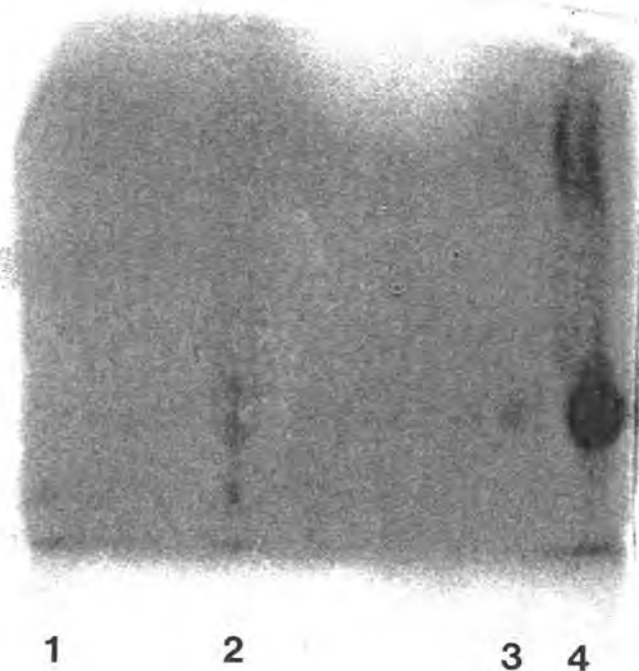
Lane 1: Crude aqueous *A. bisporus* tyrosinase.

Lane 2: *S. lividans* (pIJ702) pH 7.2, MMT-induced pure tyrosinase.

Lane 3: *S. lividans* (pIJ702) pH 7.2, MMT-induced impure tyrosinase.

Lane 4: Crude dried *A. bisporus* tyrosinase.

Lane 5: Commercial dried tyrosinase.



- Lane 1:** *S. lividans* (pIJ702) pH 7.6, MMT-induced impure tyrosinase.
- Lane 2:** *S. lividans* (pIJ702) pH 7.6, MMT-induced tyrosinase, 60% pellet.
- Lane 3:** *S. lividans* (pIJ702) pH 7.6, MMT-induced pure tyrosinase.
- Lane 4:** Crude aqueous *A. bisporus* tyrosinase.

No peaks were detected on analyzing a pH 7.2 intracellular extract by capillary-GC (SE 30 column) (Figure 4.20). No zone separation was seen in the extracellular extract. As in figure 4.17, an unidentifiable peak was detected at 3.9 minutes on analysis of this sample. (Standards: see section 4.2.2.4).

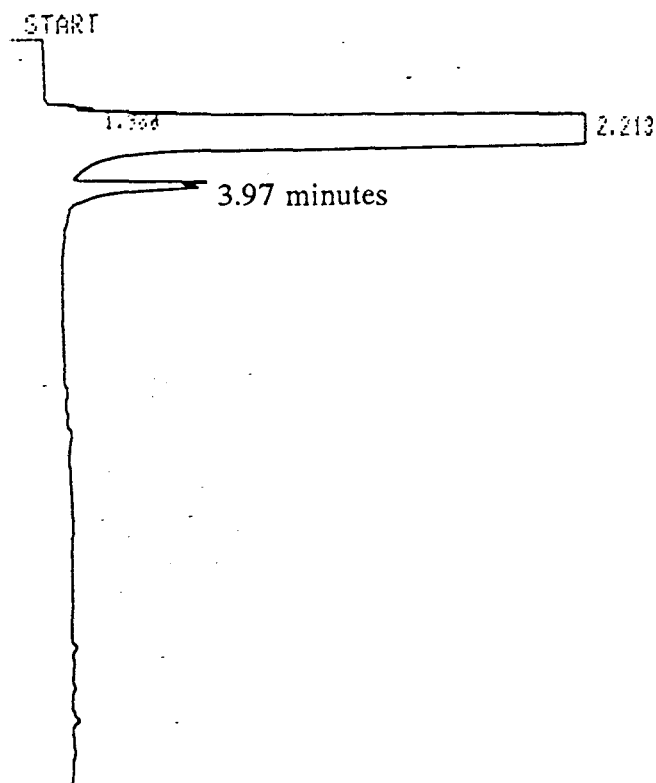


Figure 4.20: Extracellular extract of *S. lividans* (pIJ702) in pH 7.2 MMT medium (homogeneous sample).

4.2.2.6. Enzyme production in pH 7.6 GYM and MMT media

Due to relatively high enzyme production levels previously noted at pH 7.6, *S. antibioticus*, *S. glaucescens* and *S. lividans* (pIJ702) were grown in GYM and MMT at this pH in an attempt to optimize enzyme production.

No colour change, biomass production or enzyme activity was detected in GYM media flasks. As before, a distinct colour change was seen in MMT flasks. *S. lividans* (pIJ702) culture media changed colour after 24 hours, and *S. antibioticus* and *S. glaucescens* after two days. By day 3 a black mass (spores/precipitate) had formed on the bottom of *S. antibioticus* and *S. glaucescens* flasks. There was some difficulty in extracting intracellular tyrosinase due to the formation of this thick precipitate. After 6 days, media from all shaker-flasks was black, with *S. lividans* (pIJ702) being the darkest and *S. glaucescens* the lightest.

Figures 4.21, 4.22 and 4.23 demonstrate the relationship between biomass formation, glucose utilization and pH changes over the course of the reaction for *S. antibioticus*, *S. glaucescens* and *S. lividans* (pIJ702) respectively. Figure 4.24 compares the biomass production by these three cultures.

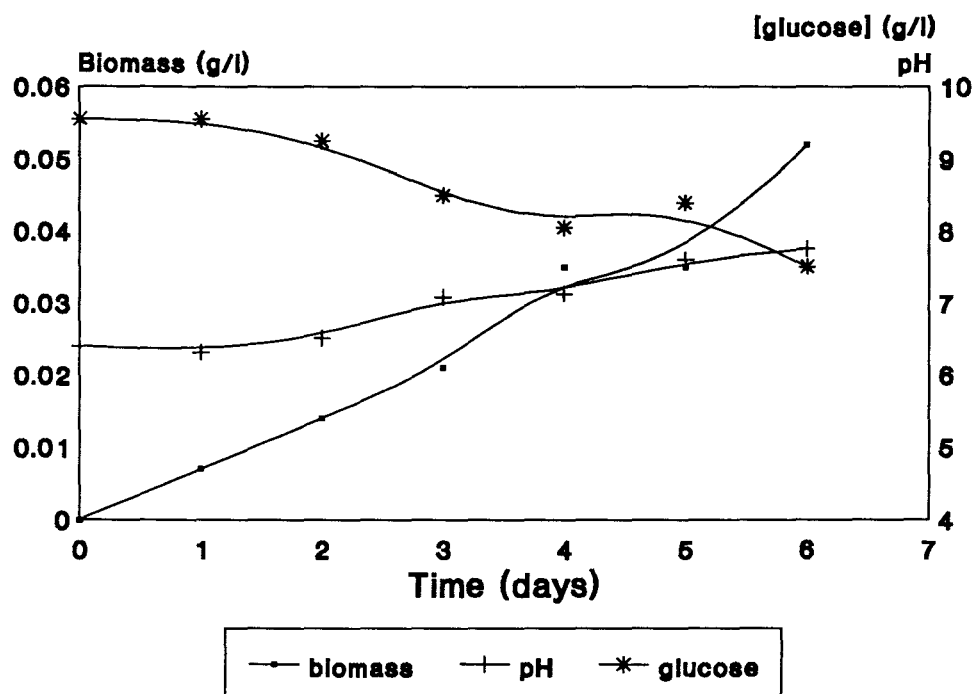


Figure 4.21: *S. antibioticus* in pH 7.6 MMT medium.

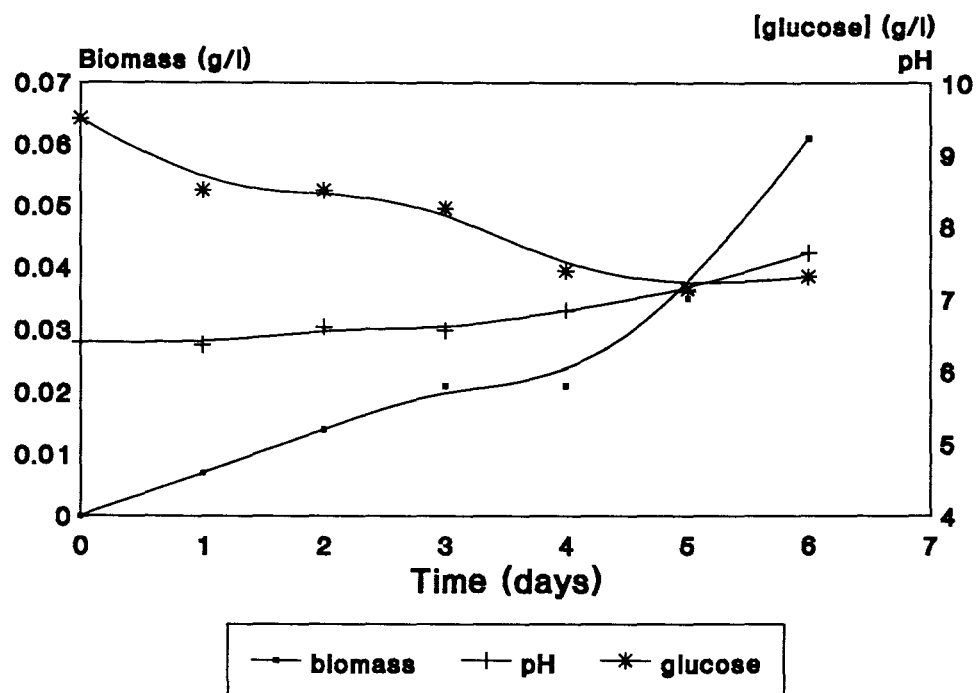


Figure 4.22: *S. glaucescens* in pH 7.6 MMT medium.

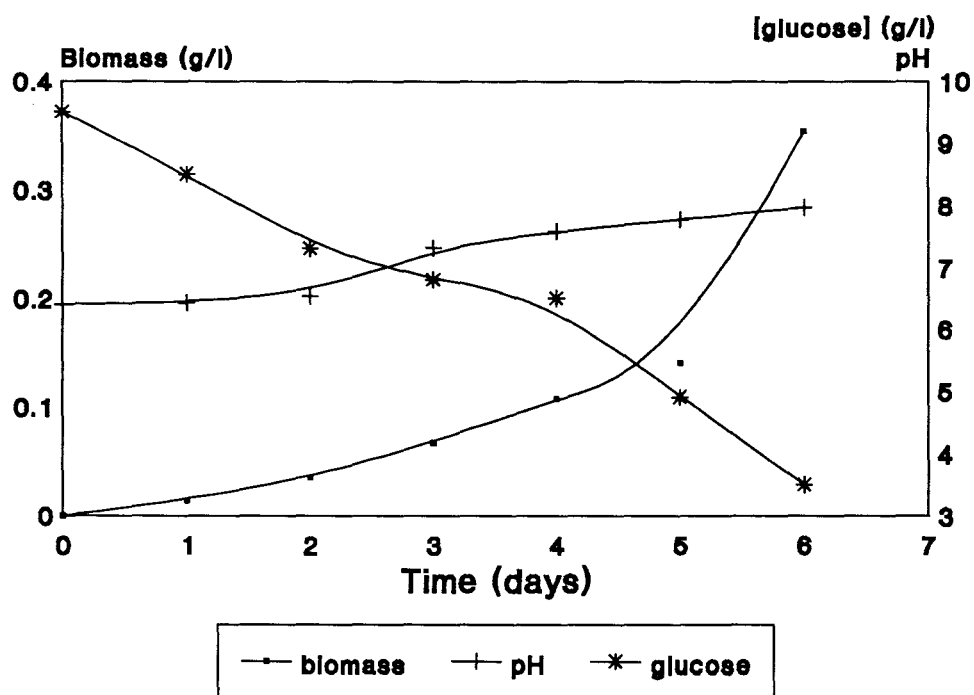


Figure 4.23: *S. lividans* (pIJ702) in pH 7.6 MMT medium.

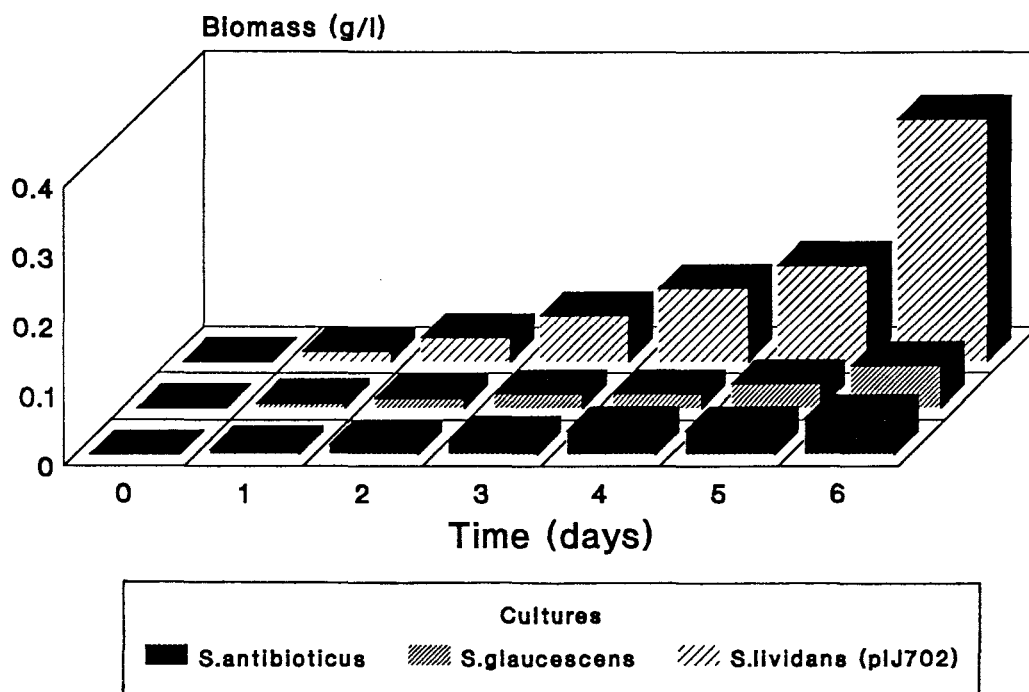


Figure 4.24: Biomass production in pH 7.6 MMT media.

MMT media pH was shown to change on the addition of casaminoacid sterilized solution. The pH of minimal media was initially adjusted to pH 7.6, autoclaved and on the addition of casaminoacids, changed to pH 6.4. The control experiment remained at pH 6.4 throughout the reaction. pH fluctuations are expected during an enzyme reaction due to the varying buffering capacities displayed by substrates and product (Cheetham, 1986).

No enzyme was seen in *S. antibioticus* extracts. Table 4.9 lists the enzyme activities of *S. antibioticus* and *S. glaucescens*.

Table 4.9: Tyrosinase activities (units of activity.ml⁻¹) in pH 7.6 MMT cultures assayed against L-DOPA.

Day	<i>S. glaucescens</i>		<i>S. lividans</i> (pIJ702)	
	Extracellular	Intracellular	Extracellular	Intracellular
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	minimal	0	0	0
5	minimal	0	5.5	minimal
6	minimal	minimal	12.2	minimal

As insufficient material was available for protein determinations, 40µl samples were loaded onto zymograms for analysis. Only *S. lividans* (pIJ702) day 6 intra- and extracellular extracts showed any colour development on gels.

4.2.3. Zymogram analysis of *S. lividans* (pIJ702) extracts

Calculation of R_f values for bands on zymograms allowed a comparison of banding patterns between the different preparations under analysis - see table 4.10. Values were calculated according to the following formula:

$$R_f = \frac{\text{the distance moved by the solute}}{\text{the distance moved by the solvent front}}$$

Table 4.10: R_f values for bands of a variety of *S. lividans* (pIJ702) tyrosinase preparations resolved on zymograms.

PLATE NO.	R_f VALUES				
	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5
4.3	0.51 0.65	0.27 0.55	0.27 0.55		
4.4	0.55	0.76	0.55		
4.5	0.30 0.67	0.57	0.57	0.43 0.74	0.74
4.6	0.93	0.70 0.79 0.93	0.74	0.25 0.74	

- Plate 4.3 - lane 1: impure pH 8 (MMT) *S. lividans* (pIJ702).
 - lane 2: aqueous crude tyrosinase.
 - lane 3: concentrated crude tyrosinase.
- Plate 4.4 - lane 1: impure pH 7.2 (MMT) *S. lividans* (pIJ702).
 - lane 2: aqueous crude tyrosinase.
 - lane 3: pure pH 7.2 (MMT) *S. lividans* (pIJ702).
- Plate 4.5 - lane 1: aqueous crude tyrosinase.
 - lane 2: pure pH 7.2 (MMT) *S. lividans* (pIJ702).
 - lane 3: impure pH 7.2 (MMT) *S. lividans* (pIJ702).
 - lane 4: concentrated crude tyrosinase.
 - lane 5: commercial tyrosinase.
- Plate 4.6 - lane 1: impure pH 7.6 (MMT) *S. lividans* (pIJ702).
 - lane 2: 60% pellet (sample obtained on salt fractionation) pH 7.6 *S. lividans* (pIJ702).
 - lane 3: pure pH 7.6 (MMT) *S. lividans* (pIJ702).
 - lane 4: crude tyrosinase (40% supernatant - sample obtained on salt fractionation).

4.3. DISCUSSION

4.3.1. Growth on solid substrate (plate tests)

The tyrosine-supplemented agar plate method gives a positive indication of tyrosinase activity. Melanin positive, tyrosinase-secreting colonies are surrounded by a brownish-black pigment, while melanin negative mutants do not discolour media containing tyrosine, even if the level of intracellular tyrosinase is high. It therefore appears that this method may be specific for extracellular, tyrosinase-secreting (*mel*⁺) producers only. Plate 3.3 is

therefore the first indication in this study of extracellular tyrosinase production by *S. lividans* (pIJ702), reportedly mainly an intracellular enzyme producer (Katz *et al.*, 1983). The gene *tye* codes for tyrosinase, and *mel* influences the extracellular activity of the enzyme. The three cultures on plates 3.1 - 3.3 are therefore *tye*⁺, *mel*⁺. The distribution of pigment (eumelanin) away from the colonies may be due to products such as L-DOPA or soluble melanin components that diffuse away from the bacterial colonies into the agar medium to undergo further polymerization (della-Cioppa *et al.*, 1990).

The tyrosinase of *S. antibioticus* and *S. glaucescens* has been reported to be inducible, with *de novo* enzyme synthesis taking place on addition of an inducer such as L-methionine (Baumann and Kocher, 1974). *S. lividans* (pIJ702) tyrosinase gene is constitutively expressed (Gardner and Cadman, 1990). As inducers were not added to the agar medium of *S. antibioticus* and *S. glaucescens* cultures, it appears as if substrate induction may be taking place i.e. tyrosine is acting as inducer, as well as substrate. As tyrosine has a very low uptake rate of 1.3 mmol.min.mg⁻¹ and has been found to be a poor inducer of *Streptomyces* tyrosinase (Baumann and Kocher, 1974), it is unlikely that substrate-induction is the prevailing mechanism. Lerch and Ettlinger (1972) reported that the substrates common to fungal tyrosinase oxidation i.e. p-cresol, tyrosine and catechol, are oxidized very slowly by Streptomyces. It therefore seems improbable that tyrosinase is being induced by tyrosine. A number of factors have been reported to be responsible for inducing or improving tyrosinase production in Streptomyces, e.g. tyrosinase production in *S. michiganensis* was induced by media rich in copper and low in ammonium (Held and Kutzner, 1990). This was reinforced by Katz and Betancourt (1988) who reported a correlation between active tyrosinase of *S. antibioticus* and copper concentration of the media. Other factors include the age and size of the inoculum (Katz and Betancourt, 1988), the temperature of incubation (Gardner and Cadman, 1990) and the addition of glucose (Katz and Betancourt, 1988). 2-Aminocarboxylic acids, particularly L-methionine and L-leucine, have been widely reported as the inducers of *S. antibioticus* and *S. glaucescens* tyrosinase (Baumann and Kocher, 1976; Crameri *et al.*, 1982; Hintermann *et al.*, 1985; Katz and Betancourt, 1988). As additional methionine was not added to agar media, tyrosinase production may be induced by methionine contained within the constituents (e.g. yeast extract) of the M3 substrate. Since optimum enzyme induction is

observed at 0.1mM methionine (Katz and Betancourt, 1988), methionine of media constituents may be sufficient to stimulate enzyme production.

4.3.2. Enzyme production in shaker-flask cultures

In the present study batch and fed-batch culture systems have been adapted and employed for the optimization of tyrosinase production.

4.3.2.1. Enzyme production in M3 medium

The most notable result was the production of tyrosinase in the absence of an inducer in culture media, again implying induction by amino acids contained within malt and yeast extracts of the growth medium. Tyrosine was present in the media and colour changes were seen, indicating that melanin formation had taken place. The most extreme colour change was seen by the highest producer i.e. *S. antibioticus* S1 culture. This finding was confirmed in experiment 2.4.2.2.

No colour changes were seen in B2 flasks, but tyrosinase was detected on assay. This may indicate that the colour change of media is not a reliable visual indicator of enzyme production, or that melanin formation is being repressed on passaging. On streaking B2 samples onto tyrosine-containing agar, characteristic colour changes were seen, indicating that catalytically-active tyrosinase was being produced. It therefore appears as if melanin formation is repressed by broth components of the culture media; repression may be initiated by passaging. Passaging has been reported to affect culture viability and therefore enzyme production (Kirby, pers. comm.). An incomplete enzyme reaction may have taken place as there was no indication of coloured polymerization products.

A greater increase in biomass was apparent on inoculation with broth cultures than with spores. This could be ascribed to the necessary higher inoculum volume required of the former. The enzyme production of the broth-inoculated cultures was not notably greater than spore-inoculated cultures as tyrosinase is produced during sporulation (Sugiyama *et al.*, 1990).

In this medium *S. lividans* (pIJ702) enzyme production was predominantly intracellular, while *S. glaucescens* tyrosinase was found both intra- and extracellularly. These results are consistent with published findings. *S. antibioticus* tyrosinase has been reported to be mostly extruded into culture medium (Katz and Betancourt, 1988), but this experiment showed intra- and extracellularly tyrosinase in similar proportions.

To maintain uniformity within the screening programme, phenol and tyrosine were selected as assay substrates for the *Streptomyces*. With the exception of *S. glaucescens*, phenol assay results appear to be fairly consistent, whereas there was a large variation in response to tyrosine. Tyrosine is not generally regarded as a good substrate for Streptomycete tyrosinase (Baumann and Kocher, 1974). Both tyrosine and phenol are oxidized very slowly by *Streptomyces* tyrosinase (Lerch and Ettlinger, 1972), which may lead to ambiguity. The use of controls and replicates does, however, confirm the use of these assays as a qualitative indicator of enzyme production.

The instability of the *Streptomyces* tyrosinase enzyme was evident on the reassay of frozen enzyme fractions two months after extraction. No enzyme activity was detected.

In an attempt to detect reaction intermediates and conclusively identify tyrosinase, modified aeration experiments were carried out in chloroform and samples analyzed on capillary GC. Figures 4.6 and 4.8 give a good indication of phenol removal efficiencies of *Streptomyces* tyrosinase. Concentrating enzymes by freeze-drying resulted in an increase in enzyme activity. Due to the danger freeze-drying poses to the structure of proteins composed of subunits, it may best be reserved for storage and transportation of samples (Scawen and Melling, 1986).

4.3.2.2. Enzyme production in GYM medium

The high activity of tyrosinase produced by the wild-type Streptomycetes, *S. antibioticus* and *S. glaucescens*, is shown in tables 4.3 and 4.4. These results are comparable (experiment a) and superior (experiment b) to those attained by Gardner and Cadman (1990) using the construct, *S. antibioticus* (pIJ702). A comparison of these results are presented in table 4.11.

Table 4.11: A comparison of published and experimental results.

	BIOMASS (g.l ⁻¹)	ENZYME ACTIVITY (units.ml ⁻¹)
G + C (1990) <i>S. antibioticus</i> (pIJ702)	9.0	66.0
Present study - exp. a <i>S. antibioticus</i> (10 hours, 30°C)	0.7	63.8
Present study - exp. b <i>S. antibioticus</i> (20 hours, 30°C)	1.46	500.6
Present study - exp. b <i>S. glaucescens</i> (20 hours, 30°C)	1.67	673.1

G + C (1990): Gardner and Cadman (1990)

Results show that good enzyme production can be achieved using wild type Streptomyces. This would facilitate tyrosinase production on a large scale as genetic manipulation would be unnecessary and plasmid stability not a consideration. On increasing biomass production, enzyme production was increased notably.

Enzyme production in experiment a appears to be biomass-dependent in *S. antibioticus* and *S. glaucescens*, i.e. it is directly associated to the generation of energy by the organism. Both cultures reached a particular biomass threshold level (approximately 0.6 g.l⁻¹) before the onset of enzyme production. This level was attained earlier in *S. glaucescens*. Maximum enzyme production in this culture was at 30 hours, followed by a dramatic drop in activity at 35 hours even though the biomass was still increasing. Product deactivation, possibly due to proteolytic activity, may be taking place. Enzyme production in *S. antibioticus* was still increasing exponentially by the end of the reaction. Gardner and Cadman (1990) reported maximum enzyme production by *S. antibioticus* (pIJ702) at 35 hours. This experiment therefore suggests that *S. antibioticus* is the superior tyrosinase-producer.

The two-temperature regime employed in this experiment notably increased enzyme production. Besides being linked to biomass formation, enzyme production is therefore

also temperature dependent. Enzyme activity generally increases with an increase in temperature, but this is counteracted by enzyme deactivation. The rate and extent of deactivation are thought to increase with increasing temperatures (Roels, 1983). Reducing the reaction temperature from 30° to 25°C therefore lowered the possibility of thermal deactivation of active tyrosinase.

pH appears to have a profound effect on enzyme production and enzyme stability. A report by Erpicum *et al.* (1990) on the extracellular production of β -lactamase by Streptomyces stated that enzyme production decreased with an increase in pH up to pH 9.2. It is apparent that this deactivation also applies to tyrosinase production in *S. antibioticus* and *S. glaucescens*. pH 9 cultures produced no enzyme and little biomass.

Plate 4.1 gave the first indication, in this study, of tyrosinase production by the *Streptomyces*. The instability of the enzyme is evident as repeated assays and zymogram analyses did not detect any enzyme. The extracellular *S. glaucescens* enzyme has previously been reported to be unstable (Baumann *et al.*, 1976).

Experiment b showed intra- and extracellular enzyme production by both cultures; the majority of the *S. antibioticus* enzyme was secreted. Figure 4.14 shows the association and dependence of enzyme production on biomass formation in *S. antibioticus*. The pattern is less clear in *S. glaucescens*, but factors stimulating biomass clearly increased enzyme production. The addition of methionine as enzyme inducer had little influence on enzyme production levels. Results do not indicate a requirement for additional methionine as methionine contained in media constituents was sufficient for enzyme induction. The only noteworthy difference between control and experiments is seen after 40 hours, probably once media is depleted of natural methionine. At this stage experiments show higher enzyme production than the methionine-less control.

Enzyme activities recorded in these experiments compare favourably with that of *A. bisporus* crude tyrosinase. The activity of crude mushroom tyrosinase averaged at 1 520 units of activity.ml⁻¹ when assayed against L-DOPA. The highest activity detected in the Streptomyces was that of *S. glaucescens* extracellular extract, where 670 units. ml⁻¹ activity was detected. This activity is approximately 44% that of crude *A. bisporus*

tyrosinase activity. This particular optimization system, employing a two-stage temperature regime, has therefore produced the highest Streptomyces tyrosinase levels of the current study.

4.3.2.3. Enzyme production in GYM medium using *S. lividans* (pIJ702)

As aeration was shown to be critical for Streptomyces biomass and tyrosinase production (experiment 4.2.2.2, control 2), larger 1 litre flasks were used in this experiment. This adaptation did not appear to alter biomass production significantly. Doubling the biomass production period and lengthening the culture time to 72 hours resulted in a biomass increase of approximately 8 g.l⁻¹. Despite this substantial improvement in biomass, the system employed did not induce tyrosinase production by *S. lividans* (pIJ702).

The absence of tyrosinase may be due to either one or both the following factors:

1. No thiostrepton in culture media.

As thiostrepton is responsible for maintaining plasmid stability, its absence may have detrimental effects on enzyme production. This seems unlikely as thiostrepton-deficient M3 agar plates clearly show melanin formation. Gardner and Cadman (1990) did not include thiostrepton in GYM liquid media, but only in agar media for long-term maintenance of the plasmid.

2. Ultrasonication may be unable to effectively extract intracellular tyrosinase. Studies carried out by a number of researchers (Lerch and Ettlinger, 1972; Baumann and Kocher, 1974) report the efficient extraction of tyrosinase by ultrasonication.

4.3.2.4. Enzyme production by *S. lividans* (pIJ702) in various media

A pH of 8 has been shown by Bourn (pers. comm.) to be the optimum pH for tyrosinase production by *S. lividans* (pIJ702). Only MMT media showed the colour change normally associated with enzyme production. An uninoculated control experiment did not change colour, confirming that the production of black pigment is associated with enzyme production and melanin formation. Media colour started changing after 24 hours, but

intracellular enzyme activity was only detectable after 3 days. It is possible that enzyme assays are not sensitive enough to detect the trace amounts of tyrosinase initiating media colour development. Results support published findings as only intracellular tyrosinase was detected.

The use of zymograms as a detection system for extracts of low enzyme activity was demonstrated in this study. Zymograms appear more suitable for qualitative indications of enzyme presence, while enzyme assays give a better quantitative indication of enzyme activity. Zymograms also illustrated the stability of the pH 8 *S. lividans* (pIJ702) enzyme. Lerch and Ettlinger (1972) had previously reported the stability of *S. glaucescens* at this pH. pH 8 may therefore also stabilize *S. lividans* (pIJ702). Optimum reaction conditions therefore have to achieve a compromise between maximum culture stability and maximum enzyme activity.

After centrifugation and preliminary purification of the precipitate-containing intracellular enzyme fraction, enzyme activity was lost. This may suggest enzyme association or binding to the melanin precipitate. It therefore seems essential to purify enzyme fractions immediately on extraction.

4.3.2.5. The effect of pH on *S. lividans* (pIJ702) in MMT

Due to results previously determined, cultures were harvested 5 days after inoculation and samples purified immediately upon extraction. pH 7.4 appeared to be the optimum pH for biomass production by *S. lividans* (pIJ702). Although impossible to assay the impure fraction due to the presence of an extensive precipitate, precipitate (eumelanin) development indicated strong enzyme activity. No enzyme was detected on purification, suggesting the enzyme may be bound to the black precipitate or product and lost during purification.

Two situations may exist on enzyme-precipitate binding, i.e. irreversible binding where the structure of the enzyme has been chemically modified and its activity abolished or greatly reduced, or competitive inhibition where the enzyme has a single active site to which both substrate and product bind. The active site is therefore blocked for substrate attachment

by the product. In this case both factors may be causing enzyme inactivation due to extensive product development. The pH of the medium also plays an important role as it determines the contribution of cyclization and hydroxylation through the protonation/deprotonation of the key intermediate of L-DOPA oxidation, o-dopaquinone (Rodriguez-Lopez *et al.*, 1991).

Enzyme activity appears to be linked to biomass production, with pH 8 producing the least enzyme. This correlation would have to be verified by further studies. Although pH 8 was previously selected as optimum pH (Bourn, pers. comm.), these results disprove this claim for the culture conditions utilized in this study. Enzyme production by a specific species also appears to differ quantitatively in replicate culture systems. Experiments 2.4.2.4 and 2.4.2.4 employ identical pH 8 reaction conditions, but higher activities were recorded by the former experiment. The slightest change in culture conditions or inoculum appears to affect enzyme production.

Zymograms were used to confirm enzyme presence and prove the stability of the pH 7.2 tyrosinase extract.

4.3.2.6. Enzyme production in pH 7.6 GYM and MMT media

Due to results obtained in the previous experiment, pH 7.6 was selected as the optimum pH for enzyme production. These experiments however prove that culture conditions should be optimized individually for each culture. It became apparent that culture conditions cannot be applied to all enzyme-producing strains. pH 7.6 may be suitable for the production of tyrosinase by *S. lividans* (pIJ702), but was unfit for the induction of *S. antibioticus* and *S. glaucescens* tyrosinase. While GYM medium was suitable for *S. antibioticus* and *S. glaucescens* enzyme production, it proved inadequate for the production of tyrosinase by *S. lividans* (pIJ702). High biomass and tyrosinase production was found in *S. lividans* (pIJ702) MMT cultures, but not in the remaining two cultures.

Inconsistencies in enzyme production by a specific species in replicate culture systems was reported in the previous experiment. This is also valid for *S. lividans* (pIJ702) in pH 7.6 MMT. Diminished levels of tyrosinase were seen both extra- and intracellularly.

A number of factors may be responsible for these reduced levels. Analyses showed a daily pH increase linked to biomass production. As a 10-fold increase in biomass production was seen, a much higher pH would be expected. A correlation between increasing pH and reduced enzyme production has previously been demonstrated in MMT. In general, enzymes are active over only a limited pH range. As enzymes possess many ionizable groups, pH changes may alter the conformation of the enzyme, the binding of the substrate, and the catalytic activity of the groups in the active site of the enzyme (Cheetham, 1986).

Enzyme production was not detected in *S. antibioticus* cultures. This is consistent with published findings as methionine is the only known inducer of *S. antibioticus* tyrosinase and growth media did not contain any methionine sources. A colour change indicating enzyme and melanin production was seen in culture media, suggesting that low levels of enzyme induction may have been stimulated, possibly by other amino acids such as L-leucine or L-phenylalanine. Tyrosinase was not detected on assay or zymograms, either indicating very low enzyme levels or enzyme inhibition by product (eumelanin) binding. Extensive precipitate development had occurred.

4.3.3. Zymogram analysis of *S. lividans* (pIJ702) extracts

Crude mushroom tyrosinase was included on each gel as a positive standard. pH 7.2 extracts repeatedly showed a single tyrosinase band, whereas multiple bands were present in pH 7.6 and pH 8 fractions. It appears as if tyrosinase production at pH 8 induces some change in enzyme structure as two distinct bands are seen. These forms may have resulted due to activities such as processing or post-translational modification or pH-induced changes to the tertiary structure of the protein enzyme. One or more of these factors may contribute to the presence of two tyrosinase forms.

On purification of the pH 7.6 tyrosinase extract (Plate 4.6) the single enzyme (lane 1) appears to disintegrate into three catalytically active components (lane 2). The pure pH 7.6 sample is again a single enzyme fraction. It is unlikely that isozymes form on purification, but probable that a single enzyme disintegrates into three enzymatically active fractions.

4.3.4. Enzyme induction by methionine

The induction of *S. antibioticus* and *S. glaucescens* tyrosinase by methionine has been previously reported (Hintermann *et al.*, 1985; Katz and Betancourt, 1988; Betancourt *et al.*, 1992). In a number of experiments carried out in this study, the addition of methionine proved unnecessary for enzyme induction. This suggests that either the enzyme is constitutively expressed, another inducer is present, or the methionine content of growth medium components is sufficient for enzyme induction. The third option appears to be most feasible due to published results of induction studies. It seems plausible that enzyme induction may also depend on the particular culture system and culture medium employed for enzyme optimization.

It is, however, interesting to speculate on the mechanism by which methionine might induce the tyrosinase enzyme. Information on methylation has been derived from studies using *Escherichia coli* K-12 and its related bacteriophages (Sternberg, 1985). The method by which methionine induces tyrosinase production in *Streptomyces* may therefore follow a similar approach.

The expression of certain genes in response to methionine addition to *E. coli* culture media may involve the methylation of certain nucleotide bases in DNA by specific methylases, viz: DNA adenine methylation (*dam*) or DNA cytosine methylation (*dcm*). *Dam* recognizes the 5' -GATC- 3' sequence and its affect on gene expression can be direct, i.e. via the methylation of regulatory sites, or indirect (Marinus, 1987). It has been proposed that *dam* methylation of specific -GATC- sequences may be needed to regulate gene expression or to affect the initiation of chromosomal replication (Katz and Betancourt, 1988).

Inspection of the *S. antibioticus* nucleotide sequence of the *mel* fragment revealed the presence of four -GATC- sequences in the 5' region containing the putative promoter, and additional -GATC- sequences downstream in ORF438. A *dcm* site (-CCAGG-) also overlaps the ribosome binding site 5' to ORF438. Inspection of the nucleotide sequence of the putative promoter of the *S. glaucescens mel* operon also shows the presence of -GATC- sequences. Katz and Betancourt (1988) proposed that the methylation of such

sequences may prove important in the regulation of secondary metabolite formation, cellular differentiation and enzyme induction.

The stimulation of β -lactam synthetase production by *Cephalosporium acremonium* on the addition of methionine or norleucine, seems due to a regulatory effect of the amino acid. Methionine and its analogues appear to have a variety of regulatory roles, including enzyme induction, decreased inactivation of the enzyme or a shift in morphology to some form which contains higher levels of the enzyme (Sawada *et al.*, 1980).

It is possible that a situation similar to that proposed for the bacteriophage Mu *mom* gene by Sternberg (1985) exists in *Streptomyces*. A repressor protein attaches to the unmethylated region of the *mom* promoter and prevents transcription of the gene. Methylation therefore protects the DNA by stabilizing it or inducing a DNA conformation that prevents access of the repressor protein to the site.

4.4. CONCLUSIONS

A number of conclusions can be drawn from the results obtained in this study:

1. The development of black pigment on media supplemented with tyrosine indicates the presence of melanin-producing, tyrosinase-secreting colonies.
2. Tyrosinase production in *Streptomyces* appears to be associated with biomass production.
3. With the exception of pH 7.2 and pH 8 MMT-induced *S. lividans* (pIJ702) tyrosinase, *Streptomyces* tyrosinase appears to be very unstable.
4. When methionine-containing constituents are included in media, no additional methionine is necessary for enzyme induction.
4. The DOPA assay system is superior to assay methods using phenol or tyrosine as substrate, due to the slow oxidation of these substrates by *Streptomyces* tyrosinase.
6. Zymograms and enzyme assays should be used together for an accurate qualitative and quantitative assessment of enzyme activity.

7. High levels of tyrosinase production was seen in *S. antibioticus* and *S. glaucescens* pH 7.2 GYM media cultures using a two-temperature strategy. The highest activity recorded was 44% that of *A. bisporus* crude tyrosinase activity.
8. The absence of thiostrepton in *S. lividans* (pIJ702) GYM culture media did not appear to be responsible for the absence of enzyme production. Thiostrepton is included in media for the maintenance of long-term plasmid stability.
9. The release of intracellular *S. lividans* (pIJ702) tyrosinase may be pH-related.
10. There is some evidence of pH-induced enzyme changes to pH 8 MMT-induced *S. lividans* (pIJ702) intracellular tyrosinase.
11. On purification of pH 7.6 MMT-induced *S. lividans* (pIJ702) tyrosinase, the single enzyme disintegrates into three enzyme sub-types of unknown nature.
12. Tyrosinase appears to be bound and inhibited by its association with the melanin product or precipitate. Melanin formation is extensive in MMT, and limited in GYM media. The exclusion of tyrosine from liquid media would prevent excessive melanin formation and subsequent enzyme inhibition.
13. It is possible that reduced intracellular enzyme activities may be due to inefficient extraction, as tyrosinase is cell-bound.
14. Held and Kutzner (1990) reported the induction of tyrosinase in *S. michiganensis* by the addition of copper. This absolute copper requirement has not been shown for the cultures used in this study, but Katz and Betancourt (1988) state that the synthesis of catalytically active tyrosinase is dependent on copper. Maximal enzyme production is found at 0.5 - 5 $\mu\text{g}.\text{ml}^{-1}$ $\text{CuSO}_4.5\text{H}_2\text{O}$.
15. Enzyme production in *Streptomyces* is pH dependent. Maximum activity is a function of pH when monophenols and o-dihydroxyphenols are used as substrates (Lerch and Ettlinger, 1972).
16. Optimum culture conditions cannot be applied to all tyrosinase-producing *Streptomyces*, but should be investigated for each individual culture.
17. Melanin has been produced in recombinant *Escherichia coli* from a cloned tyrosinase gene. The advantage of tyrosinase production in *Streptomyces* is their ability to secrete proteins.
18. Disadvantages of *Streptomyces* tyrosinase include the apparent instability of the enzyme and its proposed inhibition by product (eumelanin) binding.

- a. The enzyme is secreted.
- b. Tyrosinase is a single, monomeric enzyme.
- c. There is no evidence of enzyme multiplicity.
- d. Streptomycetes are natural permissive hosts for genetic studies.
- e. The wild-type *S. antibioticus* and *S. glaucescens* strains are good enzyme producers.
- f. Enzyme production has been optimized in a simple fed-batch liquid culture system.
- g. Large quantities of enzyme can be produced in simple media.
- h. The Streptomycetes can be manipulated by standard microbiological techniques.
- i. Streptomycetes are hardy spore-producers.
- j. For the above reasons, tyrosinase production by Streptomycetes requires further investigation as a potential commercially viable source of the enzyme.

CHAPTER 5

Agaricus bisporus TYROSINASE

Summary Investigations of *Agaricus bisporus* tyrosinase verified enzyme stability and activity over a range of pH values and temperatures. Enzyme kinetics were compared on monophenol and o-dihydroxyphenolic substrates. Zymograms were evaluated as a detection system for tyrosinase activity and proved successful in aqueous media, but not in organic systems. Phenol removal studies showed the presence of an unusual binding-release system during oxidative catalysis. Alternative sources of the mushroom enzyme were also investigated, including deep-liquid fermentation of mycelial cultures for tyrosinase production.

5.1. INTRODUCTION

The screening programme instituted in Chapter 3 failed to yield a tyrosinase producer with activity comparable, or superior to that of *Agaricus bisporus*. The Streptomycetes were selected as the most promising alternative producers of tyrosinase with the next highest enzyme activities recorded (Chapter 4). Although the activity of *Streptomyces* tyrosinase is significantly lower than that of *A. bisporus*, the potential for large-scale enzyme production in liquid culture suggested its possible economic feasibility.

Since enzyme extracts from the various isolates studied were to be compared with *A. bisporus* tyrosinase under identical assay conditions, it was necessary to undertake similar studies on the mushroom enzyme. The enzyme is also easy to extract and manipulate and has been widely described in the literature.

This study is divided into four sections. Firstly, it covers an examination of the characteristics of the tyrosinase enzyme. Results obtained were used during screening as a reference system for comparisons with other enzyme extracts. Secondly, crude and commercial tyrosinase preparations were compared and the use of the crude enzyme in organic solvents determined.

As *A. bisporus* is an excellent source of tyrosinase, sections three and four deal with methods of alternative producing the *Agaricus* enzyme i.e. deep liquid culture of mushroom mycelia and extracting mushroom compost. Section three optimizes conditions for the cultivation of *A. bisporus* mycelia in liquid culture and determines the effect of rapid serial passage on enzyme production. The phenol removal ability of selected samples was determined by carrying out aeration experiments. Although mycelial tyrosinase has not been widely reported, Humfeld (1948) described the cultivation of *Agaricus campestris* in submerged culture. High mycelial yields were generated and used for producing mushroom soups, gravies and flavourings.

As many fungi have been found growing in compost and casing soil after the establishment of mushroom mycelia, mushroom compost was investigated as a possible source of tyrosinase. Laccase and tyrosinase production by compost microorganisms has previously been reported (Trigiano and Fergus, 1979; Fagan and Fergus, 1984). The effects of these organisms on the substratum, mushroom mycelia and fruiting bodies are largely unknown.

Mushroom cultivation

Conventional cultivation of the edible mushroom entails three stages. Firstly, the preparation of the mushroom spawn, followed by the preparation of a selective growth substrate, with the final stage being the utilization of the medium for mycelial growth and fruiting of the edible fungus, i.e. substrate inoculation with mushroom spawn. Growth of the mushroom mycelium takes place by degradation of the insoluble fractions of the substrate such as lignin, cellulose, hemicellulose, protein and microbial biomass. Studies of the extruded enzymes of the mycelia indicate that they can degrade most of the major plant-derived fractions of the compost substrate, including the phenolic degradation products from lignin (Wood, 1984).

A range of different enzymes are produced by mushrooms during cultivation. It is generally accepted that laccase is produced primarily by the mushroom mycelia and declines rapidly over a 4-5 day period immediately after the appearance of small fruiting bodies. Fruiting body development is induced by covering the mycelium-colonised compost with a casing layer of moistened peat-chalk mixture. The ambient temperature

is lowered and ventilation increased. Cycles of fruiting body development last approximately one week. Tyrosinase is produced by the mature fruiting body (Wood and Goodenough, 1977).

Studies undertaken are outlined below, together with references to the methods used.

A. *Agaricus bisporus* crude tyrosinase

1. Enzyme extraction from *A. bisporus* fruiting body - section 2.9.
2. A comparison of enzyme assay methods - section 2.11.
3. Characteristics of the crude enzyme i.e. enzyme stability, the effect of temperature and pH changes and a substrate specificity study - section 2.15.
4. Enzyme kinetics - section 2.17.
5. Protein determination and zymogram analysis - sections 2.12.1 and 2.13, appendices 6 and 7 respectively.
6. Aqueous phenol removal studies and product analysis - sections 2.18 and 2.20.
7. Enzyme catalysis in organic media and product analysis - sections 2.19 and 2.20.

B. A comparison of crude and commercial tyrosinase preparations

1. Zymogram analysis - section 2.13 and appendix 7.
2. Aqueous phenol removal study - section 2.18.

C. Deep-liquid culture of mushroom mycelia - section 2.6.

D. Mushroom compost as a source of tyrosinase - section 2.7.

5.2. RESULTS

5.2.1. *Agaricus bisporus* crude tyrosinase

5.2.1.1. Enzyme extraction

The procedure proposed by Atlow *et al.* (1984) yielded an extract of tyrosinase with an activity of 8 050 units.ml⁻¹ when assayed against L-tyrosine. Cold acetone was used to remove chromogenic substrates (Nelson and Mason, 1970).

5.2.1.2. A comparison of enzyme assay methods

Crude tyrosinase showed a strong positive reaction on all assay substrates, i.e. there was a distinct change of reaction medium colour from pale yellow to bright orange. Figure 5.1 reports a comparison of L-tyrosine, catechol and p-cresol assay patterns at 280nm, 420nm and 395nm respectively. Figure 5.2 is the L-DOPA assay result; presented separately as the reaction does not follow the same time course as figure 5.1. Assays against L-tyrosine and L-DOPA showed higher absorbances at time 0 due to rapid reaction rates on these substrates. Assay patterns were similar for all substrates; L-tyrosine and L-DOPA assays gave the strongest colour reactions. Enzyme assays were carried out in triplicate and results expressed as a mean value.

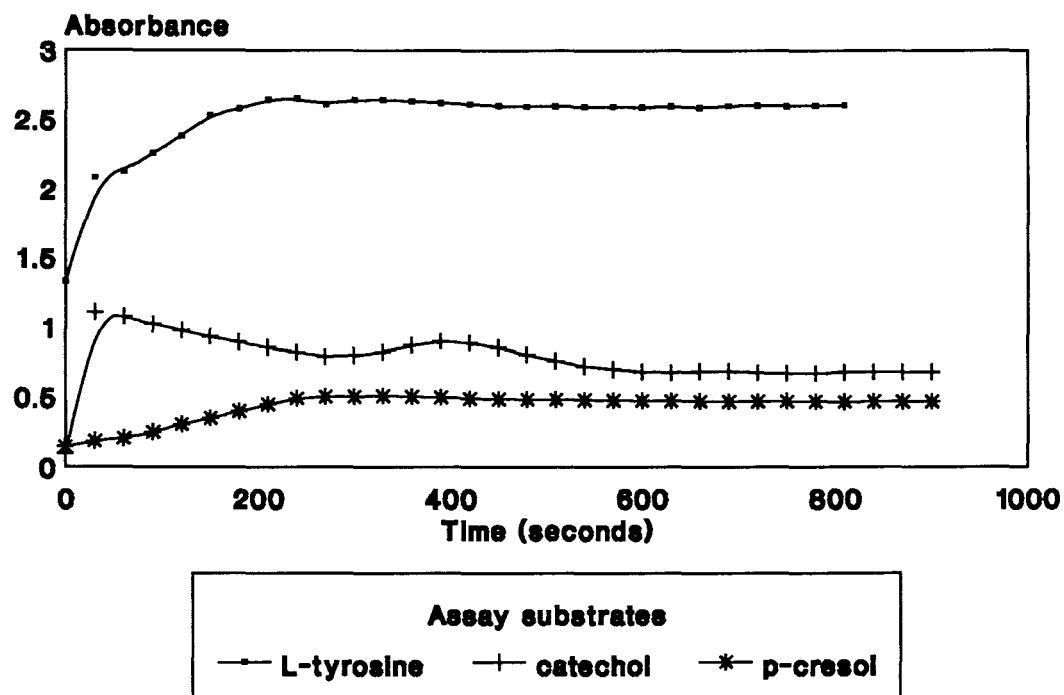


Figure 5.1: Crude tyrosinase assayed against monophenolic substrates.

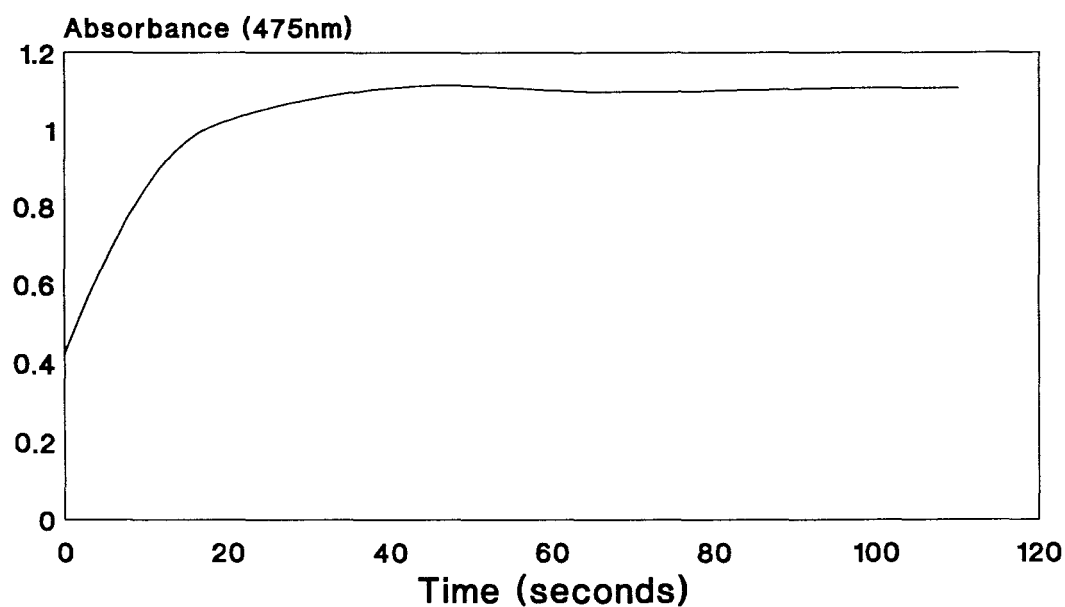


Figure 5.2: Crude tyrosinase assayed against L-DOPA.

5.2.1.3. Characteristics of the crude enzyme

All determinations were carried out using L-tyrosine as assay substrate.

Enzyme stability

It is important for an industrial enzyme to maintain good storage stability due to obvious problems associated with marketing a product of decreasing activity. For the purpose of this study the crude enzyme extract was stored at -20°C. In addition to storage at this temperature, enzyme samples were thawed before use and refrozen thereafter. General stability and the effect of constant freezing and thawing had to be determined. Results are presented in table 5.1.

Table 5.1: The stability of crude tyrosinase (units.ml⁻¹ extract).

TIME OF ASSAY	ACTIVITY
Immediately after extraction	8 050
Overnight storage at -20°C	8 167
Storage at -20°C for 5 months	10 031

Assay results throughout the course of this study repeatedly indicated the stability of the crude enzyme extract when stored at -20°C. In fact, a substantial increase in enzyme activity was seen after storage at -20°C for 5 months.

The influence of temperature

The influence of the temperature at which enzyme assays were to be undertaken was examined. Results are presented in table 5.2.

Table 5.2. The influence of temperature on enzyme activity (units.ml⁻¹ extract).

TEMPERATURE	ACTIVITY
4°C	13 940
25°C	8 050
37°C	7 490

Enzyme activity increased with decreasing temperature, indicating the versatility of the crude tyrosinase for industrial applications.

The influence of pH

A change in pH from 6-8 had no affect on enzyme activity.

Enzyme substrate specificity

The activity of crude tyrosinase was evaluated against a number of substrates. Results are presented in table 5.3.

Table 5.3: A substrate specificity profile for crude mushroom tyrosinase (units.ml⁻¹ extract).

SUBSTRATE	ACTIVITY
DL-tyrosine	1 138
L-tyrosine	8 050
L-DOPA	1 522
phenol	3 040
catechol	7 120
p-cresol	12 700

The reaction specificity of microbial enzymes is demonstrated by the increased activity of tyrosinase against the laevorotatory enantiomer, L-tyrosine, rather than its racemic form, DL-tyrosine. In addition, results show the activity of crude tyrosinase against monophenolic as well as dihydroxyphenolic substrates.

5.2.1.4. Enzyme kinetics

Michaelis-Menten kinetics were calculated for L-tyrosine, L-DOPA and p-cresol using Lineweaver-Burke plots (Figures 5.3, 5.4 and 5.5).

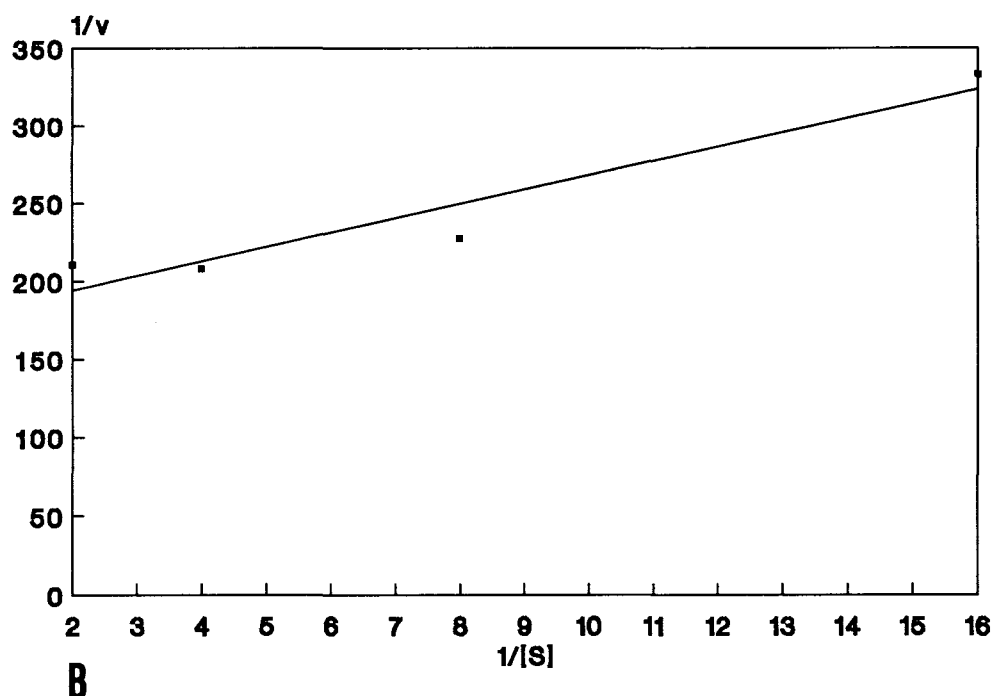
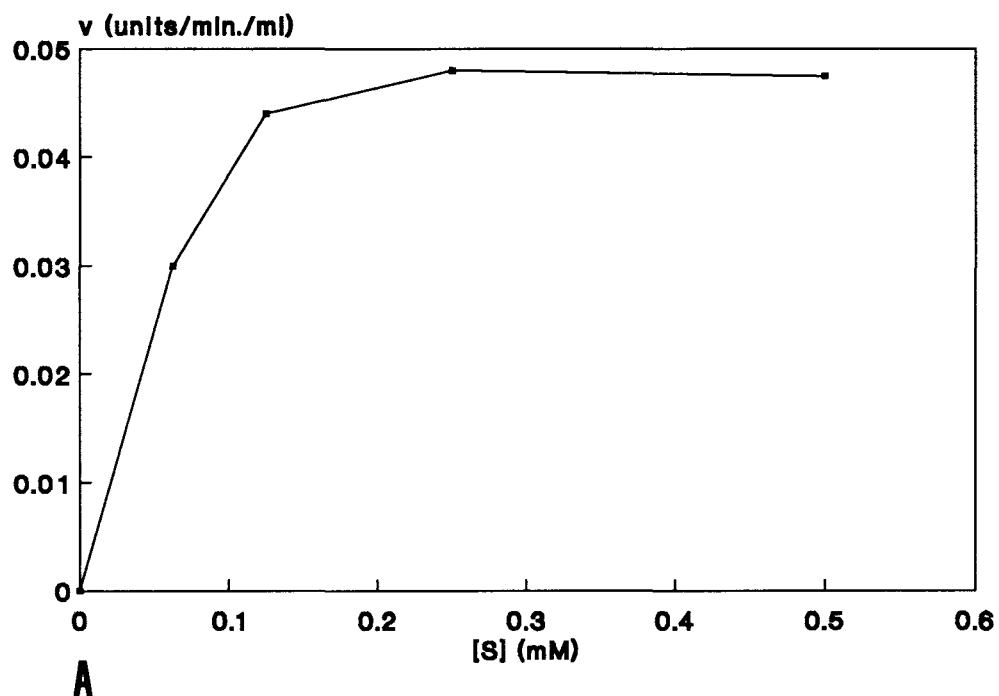


Figure 5.3: Michaelis-Menten (A) and Lineweaver-Burke (B) plots for crude tyrosinase assayed against L-tyrosine.

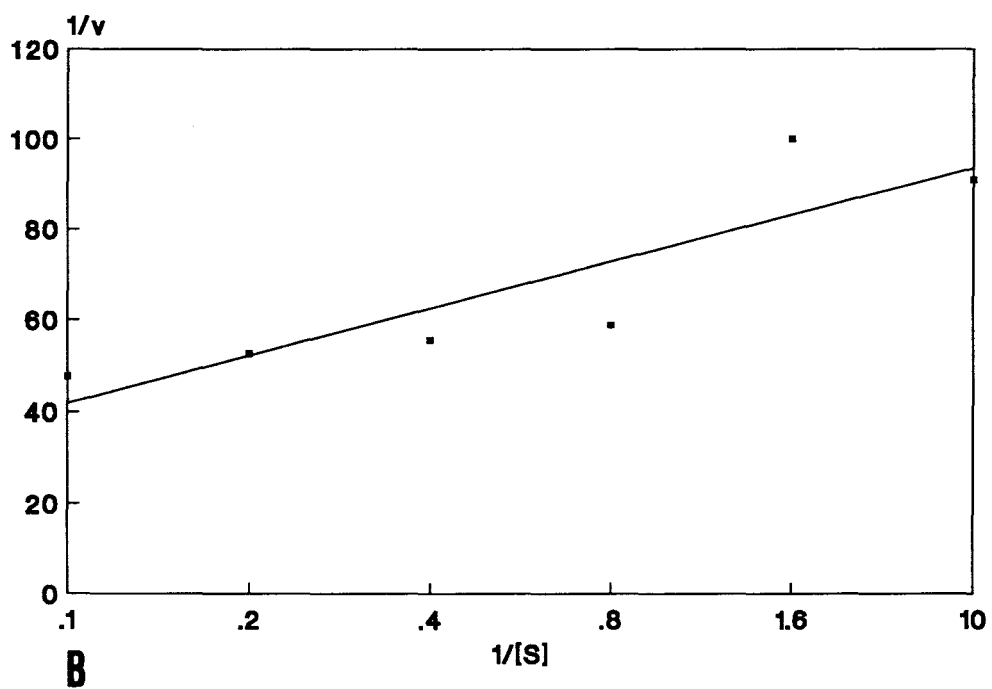
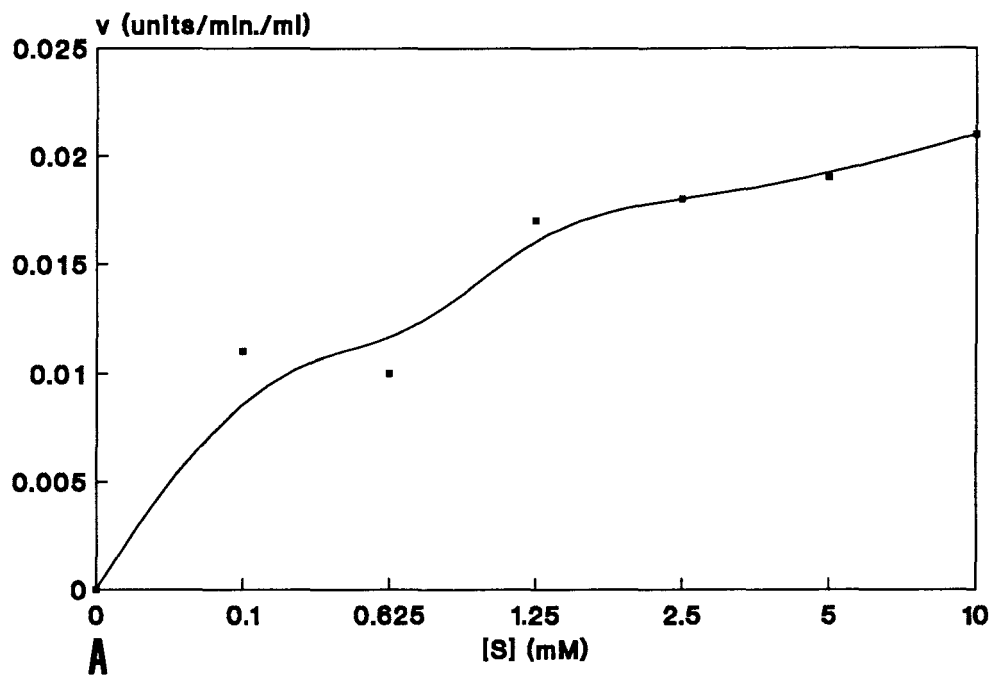


Figure 5.4: Michaelis-Menten (A) and Lineweaver-Burke (B) plots for crude tyrosinase assayed against L-DOPA.

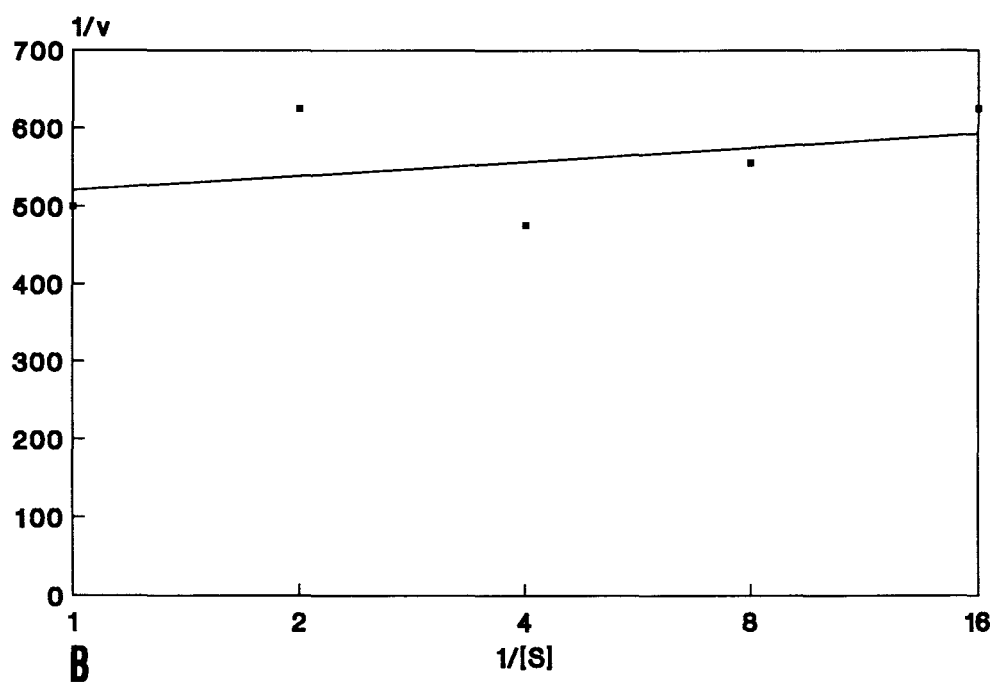
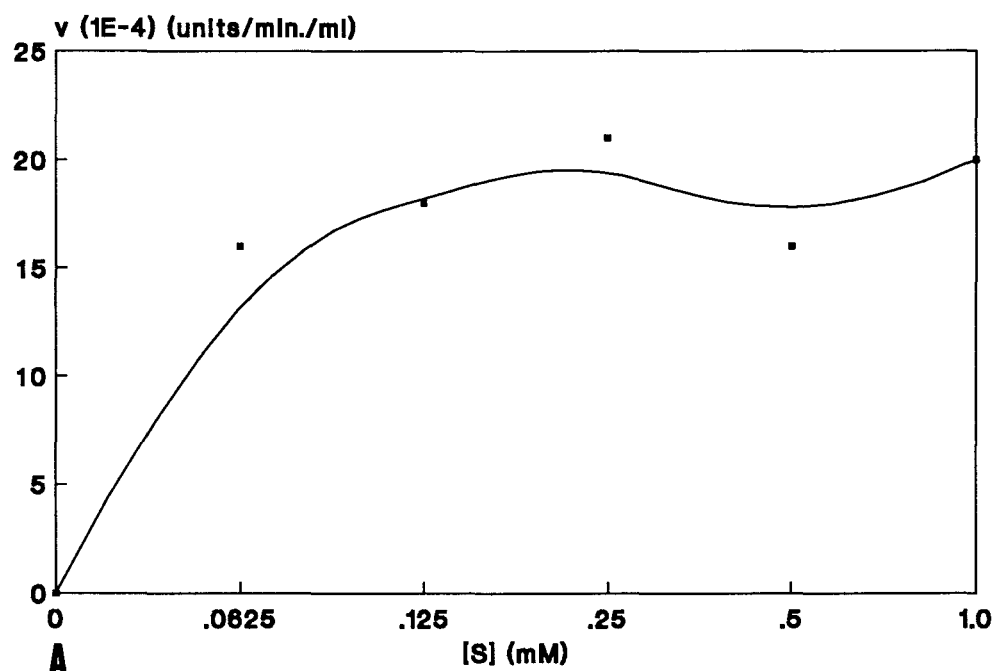


Figure 5.5: Michaelis-Menten (A) and Lineweaver-Burke (B) plots for crude tyrosinase assayed against p-cresol.

Table 5.4: Kinetic constants for crude tyrosinase on three substrates.

KINETIC PARAMETERS	SUBSTRATES		
	L-tyrosine	L-DOPA	p-cresol
$V_{\max}$ (units.min ⁻¹ .ml ⁻¹)	0.004 7	0.021	0.002
K_m (mM)	0.052	0.275	0.042

The highest rate values were those found for crude tyrosinase using L-DOPA.

5.2.1.5. Protein determination and zymogram analysis

The protein concentration of crude *A. bisporus* tyrosinase was determined by the Folin-Lowry method to be 3.75 mg.ml⁻¹. The specific activity of the enzyme against L-tyrosine (pH 6.5, 25°C) is 2 146.7 units.mg⁻¹ protein.

The crude tyrosinase extracts were run on zymograms to confirm enzyme activity and the number of active enzyme complexes present. The position of the enzyme on the gel was denoted by the deposition of brown pigment on incubation in the following substrates: L-tyrosine, L-DOPA, phenol and p-cresol. Results are presented on plates 5.1 - 5.4. Crude tyrosinase shows distinctly different patterns of activity using monophenolic and dihydroxyphenolic substrates. An extra pigment band is seen on development against L-DOPA.

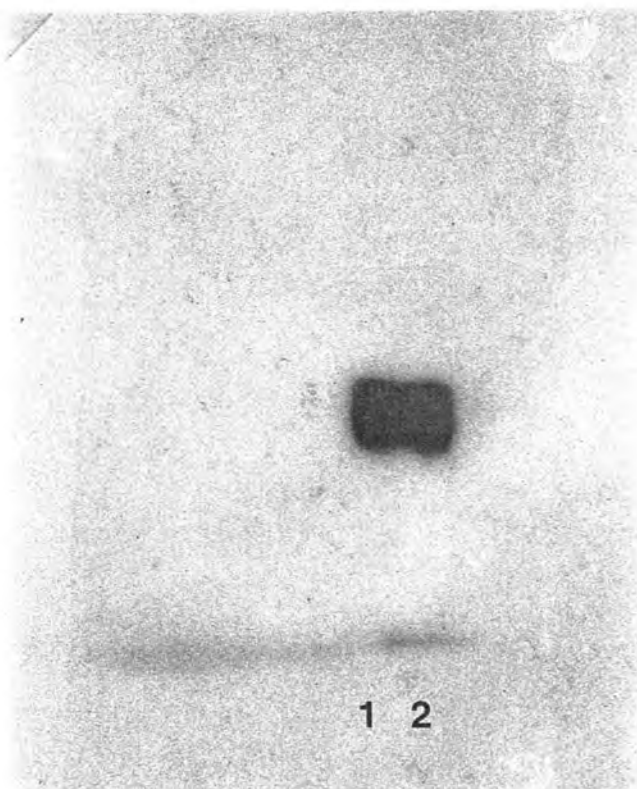


Plate 5.1: Crude tyrosinase (lanes 1 and 2) developed against L-tyrosine.

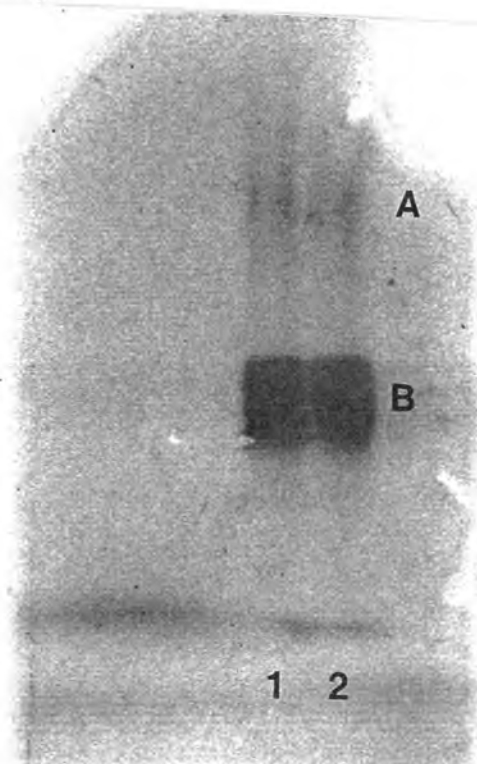


Plate 5.2: Crude tyrosinase (lanes 1 and 2) developed against L-DOPA.

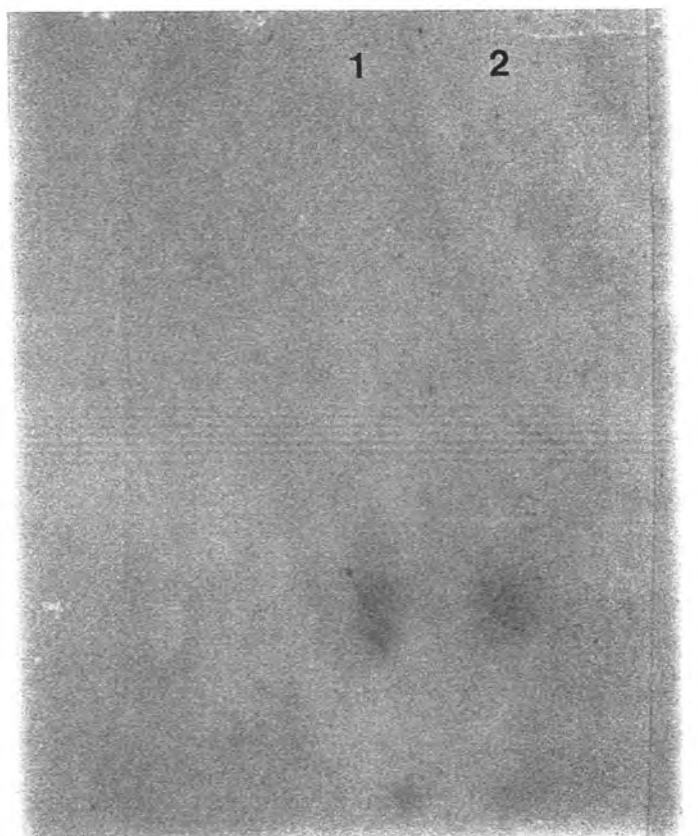


Plate 5.3: Crude tyrosinase (lanes 1 and 2) developed against phenol.

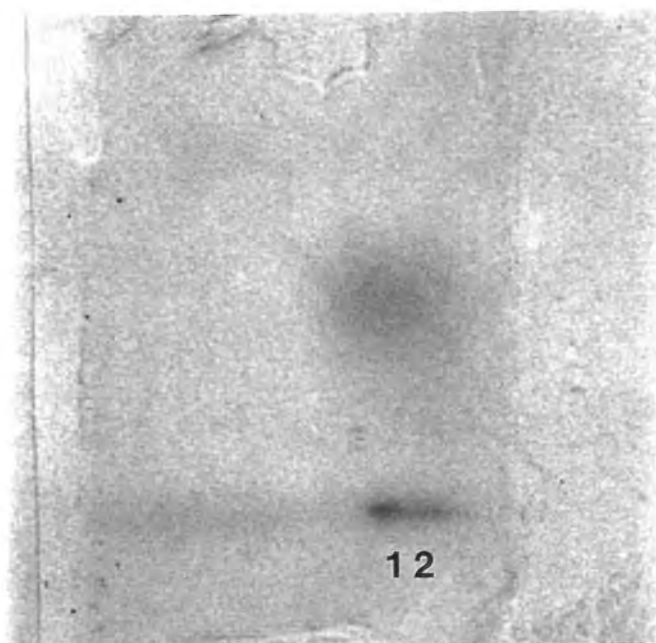


Plate 5.4: Crude tyrosinase (lanes 1 and 2) developed against p-cresol.

5.2.1.6. Phenol removal studies and product analysis

Distinct colour changes were associated with the oxidation of phenol by tyrosinase in aqueous media. At time 0 the reaction medium was light yellow due to the addition of the coloured enzyme extract. 30 seconds later the colour had changed to orange, followed by red after 4 minutes, dark red by 10 minutes and a dark purple colour after 5 hours. When left for another hour, the colour changed to brown-black and a black precipitate became clearly visible.

Samples which had been stored at -20°C before analysis, showed an inverse correlation between phenol concentration and precipitate formation. Samples 1-4 i.e. up to approximately 1.5 minutes, contained high concentrations of phenol, but little precipitate. Samples 5 and 6 i.e. 2-5 minutes, had a low phenol concentration and showed much precipitate development. Samples 7-10 i.e. 15-30 minutes, again showed a low precipitate, but high amounts of phenol. The final 3 hours of the reaction showed decreasing phenol levels with increasing polyphenolic precipitate formation.

All samples were analyzed using GC (Tenax column) and UV spectroscopy. Due to the irregularity of sampling during aqueous phenol removal studies, time had to be standardized for the graphical presentations of results and is expressed in hours.

No reaction intermediates were recorded on GC analysis. Maximum absorbances were recorded at the following wavelengths for phenolic standards:

phenol:	220nm
p-quinone:	242nm
catechol:	269nm
p-cresol:	273nm

Disparate results were seen on analysis of samples which had previously been stored at -20°C, and samples immediately analyzed - figures 5.6 (GC analysis), 5.7 and 5.8 (UV analysis). The wave-like pattern of increasing and decreasing phenol levels seen on the

GC analysis of stored samples is reminiscent of that seen in some assay results throughout the screening programme. This may again indicate phenol binding/release mechanisms.

Phenol was not detected in the last stored sample, therefore a phenol removal efficiency of 100% was achieved over 5 hours using a tyrosinase extract of activity 161 000 units.ml⁻¹. Samples analyzed immediately after sampling showed 99.3% phenol removal efficiency. Sample storage therefore seemed to have little effect on phenol removal efficiency, but did cause a fluctuation in phenol levels.

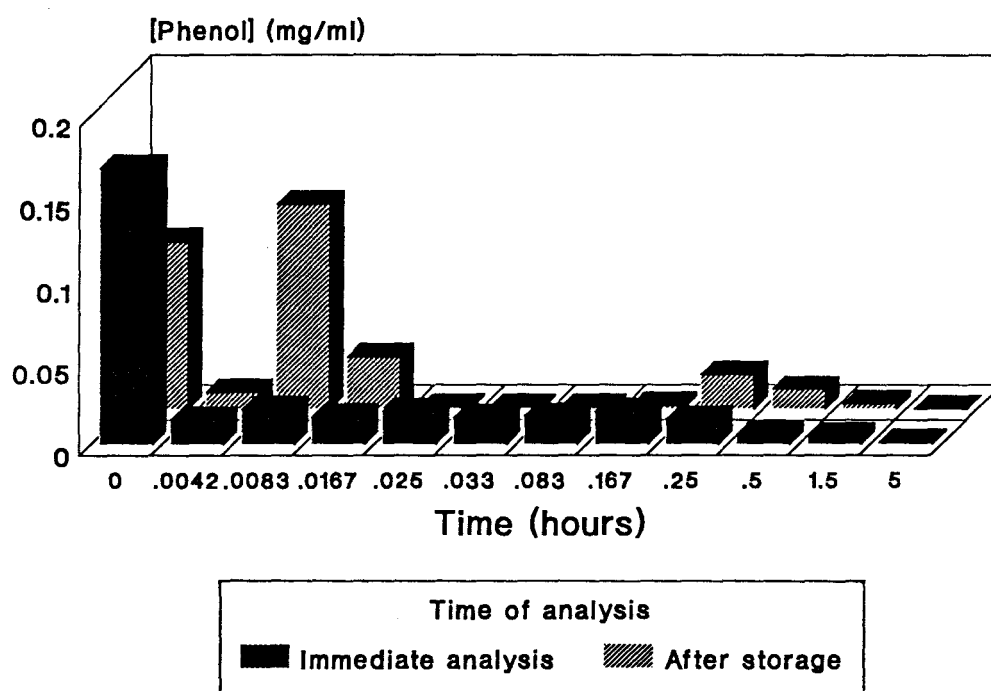


Figure 5.6: A comparison of changes in phenol concentration as determined by GC analysis for samples analyzed immediately after sampling and after storage (161 000 units crude tyrosinase added to reaction medium).

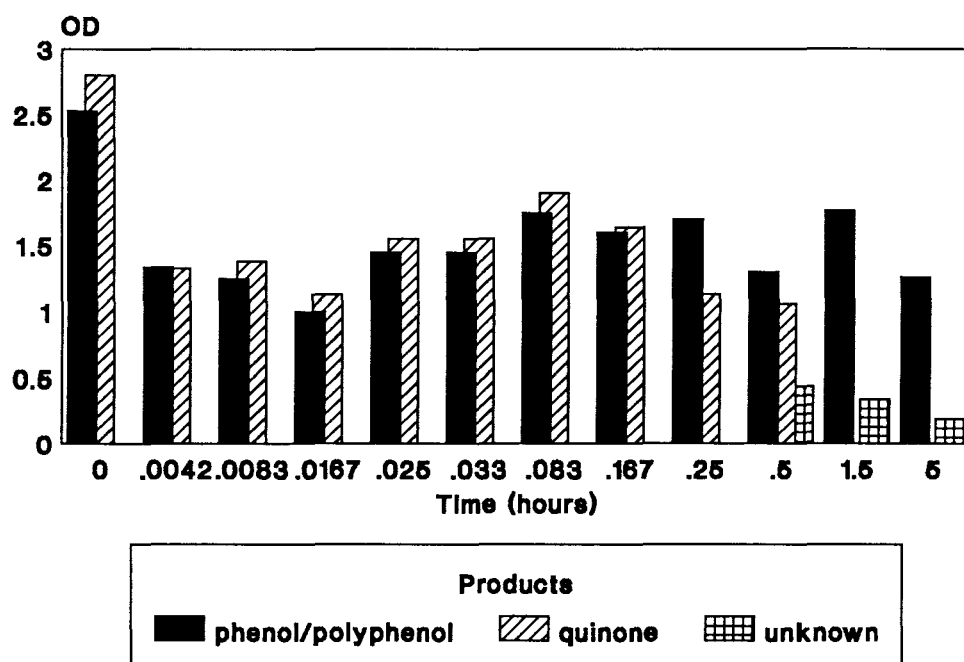


Figure 5.7: The UV analysis of samples analyzed immediately upon completion of a phenol removal experiment (161 000 units crude tyrosinase added to reaction medium).

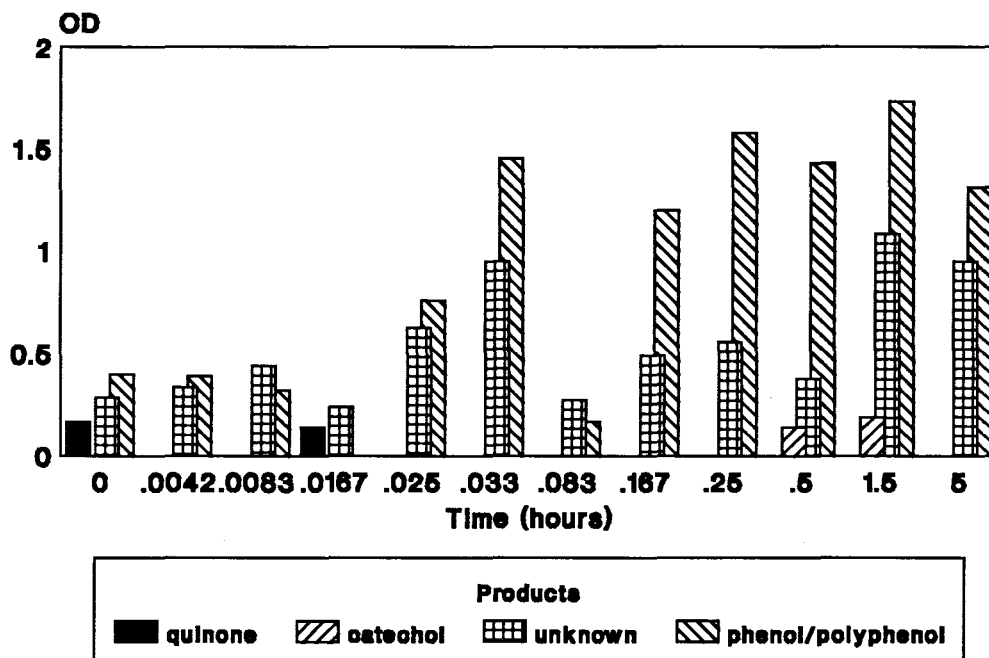


Figure 5.8: The UV analysis of samples analyzed after storage (161 000 units crude tyrosinase added to reaction medium).

Removal experiments were carried out at pH 6 and 8 to evaluate the effect of pH on phenol removal efficiency. Figures 5.9 (GC analysis), 5.10 and 5.11 (UV analysis) therefore compare the effect of media pH on phenol removal by crude *A. bisporus* tyrosinase.

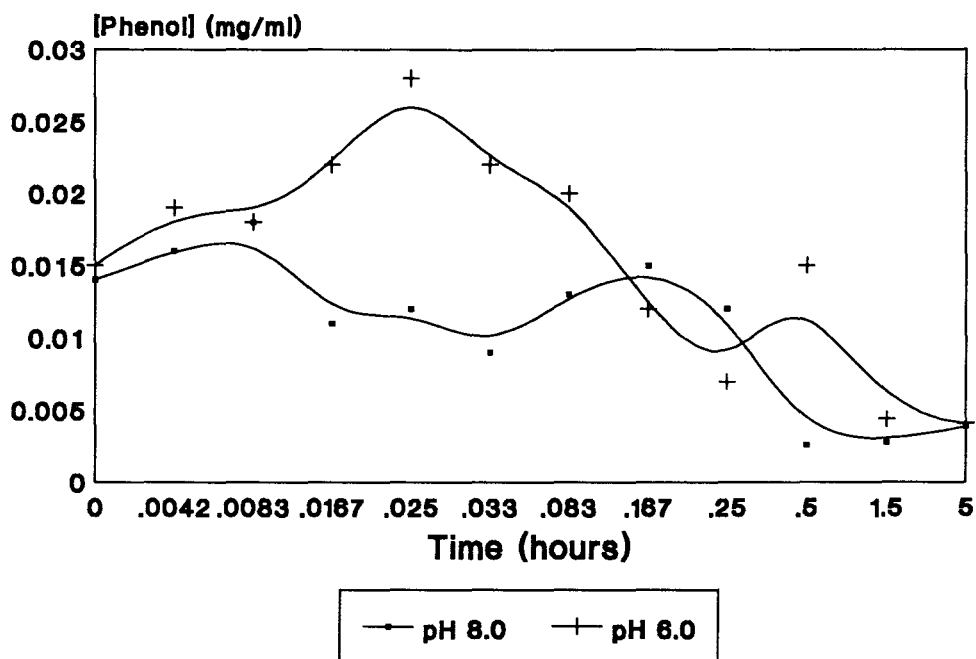


Figure 5.9: A comparison of phenol removal efficiency of 14 500 units crude tyrosinase at two different pH values (GC analysis).

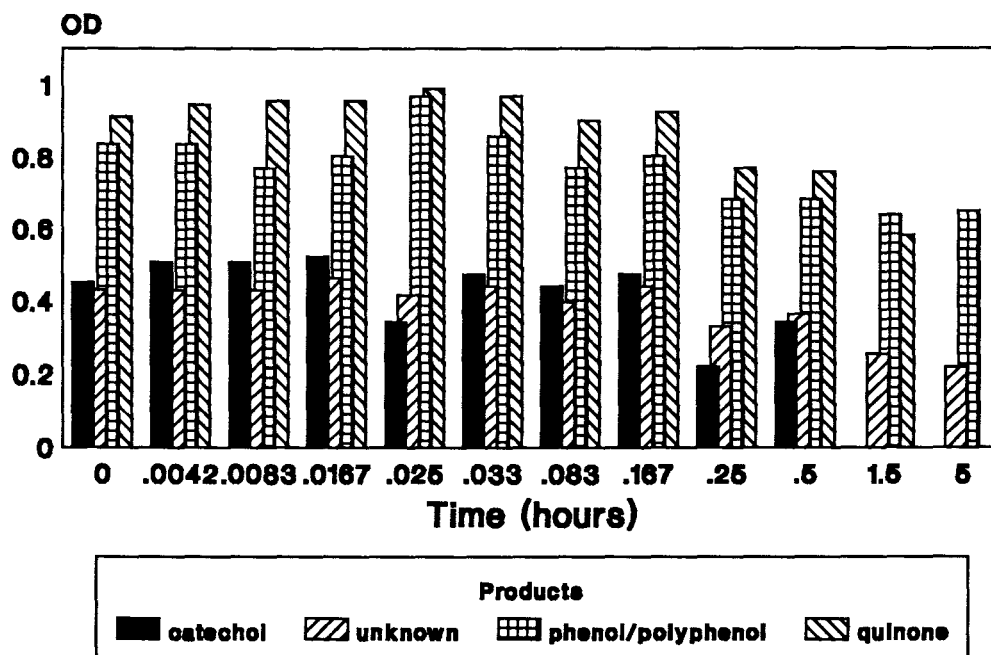


Figure 5.10: The UV analysis of samples analyzed immediately upon completion of a phenol removal experiment carried out at pH 8 (14 500 units crude tyrosinase added to reaction medium).

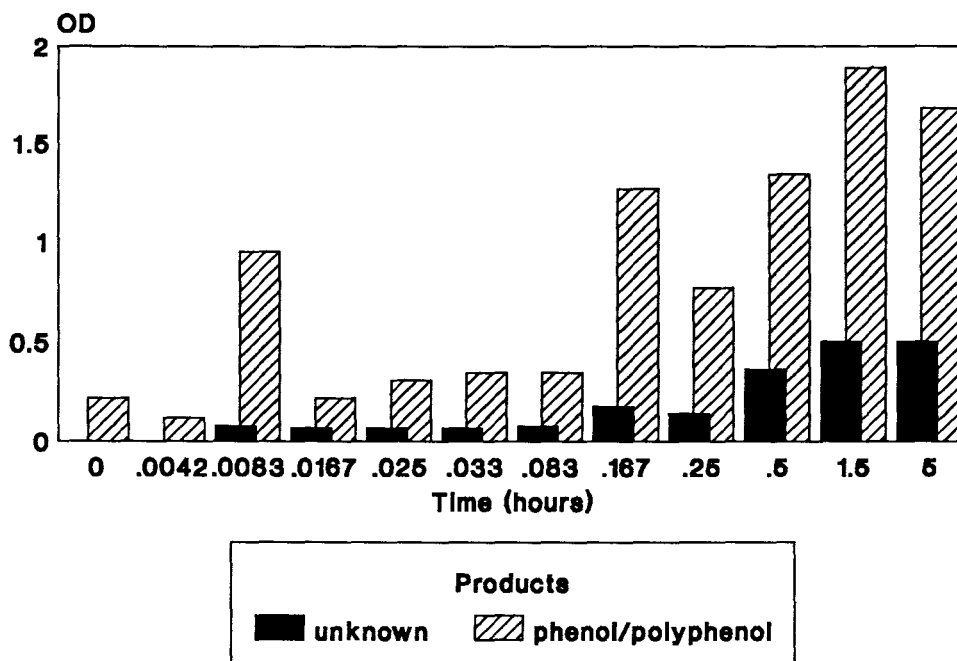


Figure 5.11: The UV analysis of samples analyzed immediately upon completion of a phenol removal experiment carried out at pH 6 (14 500 units crude tyrosinase added to reaction medium).

While the pattern of product formation (UV analysis) was affected by pH, little difference was seen in phenol removal efficiency. An enzyme extract of activity 14 500 units showed the following removal of phenol after 5 hours:

pH 8.0: 72.1%

pH 6.0: 72.7%

These results confirm preliminary findings that the enzyme can be used effectively over a range of pH values. Strict pH control during effluent treatment would therefore not be necessary.

Enzyme-product binding

Enzyme inhibition by product binding has been widely reported, but some disagreement exists as to the nature of the product responsible. Pomerantz (1964) reported catechol to be the product, while Vanni and Gastaldi (1990) implicated quinone. Both these claims were tested (section 2.21).

Upon completion of the protein-catechol dissociation experiment (section 2.21.1), sample supernatants were assayed on GC. As catechol was not detected, results suggest that protein-catechol binding probably does not take place. An alternative method was selected to test for protein-quinone binding based on the negative protein-catechol result.

The result of the protein-quinone binding experiment (section 2.21.2) is seen in figure 5.12.

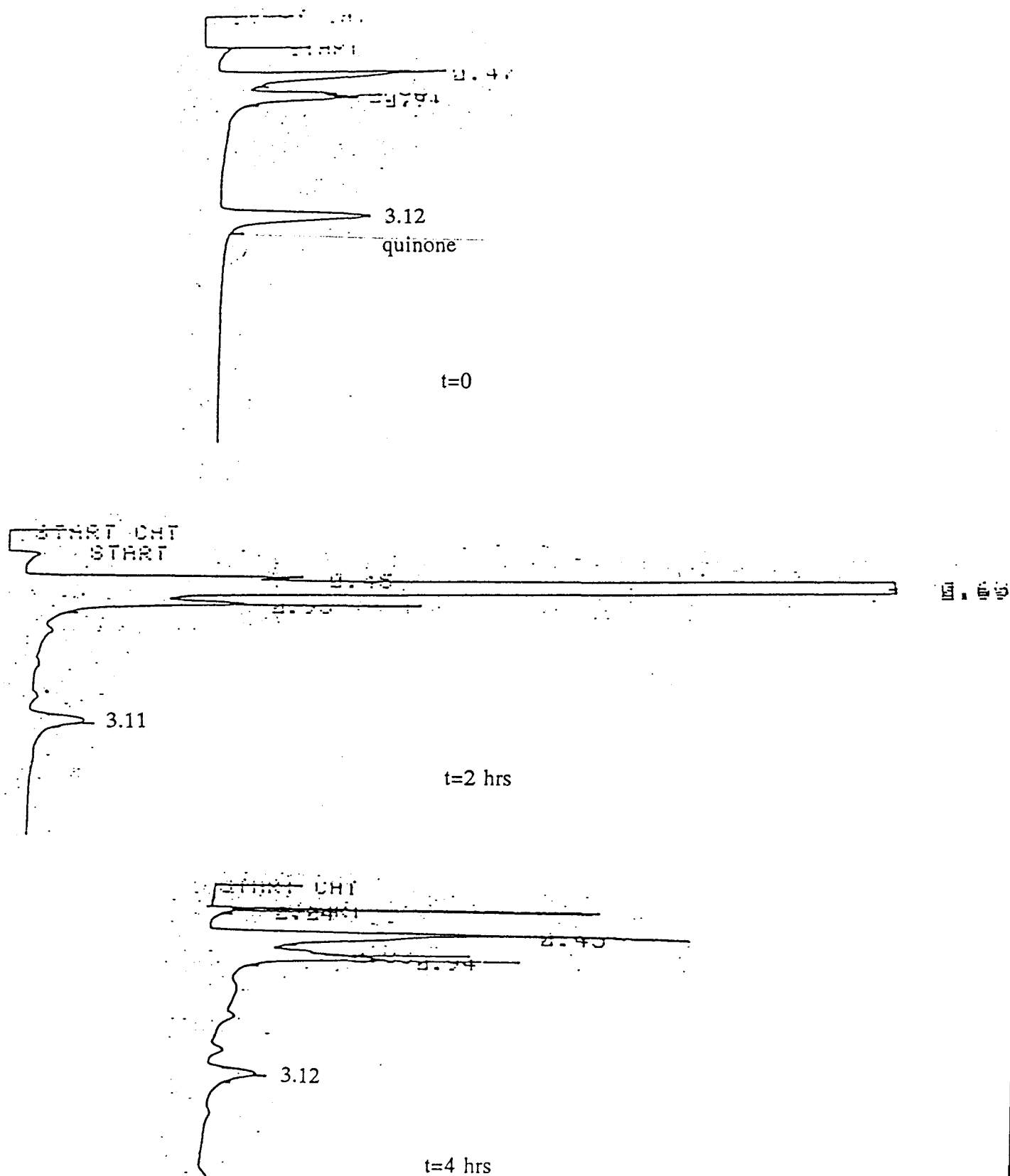


Figure 5.12: Enzyme-quinone binding - GC determination of quinone concentration after 0, 2 and 4 hours.

- A: Area = 3 800
- B: Area = 2 674
- C: Area = 913

Figure 5.12 shows quinone levels after 0, 2 and 4 hours of binding with protein. Quinone was detected, but peak area had decreased after 4 hours. Quinone was not detected on quinone-tyrosinase binding, or on mixing with aerated BSA and tyrosinase. Despite the decrease in quinone concentration seen in figure 5.12, short term results seem to indicate that non-specific protein binding alone is not responsible for the absence of quinone on product analysis. The decrease in quinone after 4 hours is to be anticipated given its spontaneous polymerization in an oxidizing environment.

Enzyme purification

In another attempt to prevent extensive enzyme-product binding and subsequent enzyme inhibition, salt fractionation studies were carried out on crude mushroom tyrosinase. Immediate removal of enzyme products or contaminating material from the enzyme active site should prolong enzyme activity and availability. Ammonium sulphate precipitation is one of the most useful procedures available for preliminary protein purification, especially when handling large enzyme volumes (Coombs and Hind, 1985). A pilot-scale fractionation experiment (appendix 8) was carried out to determine the percentage of salt required to precipitate maximum enzyme and minimum extraneous protein (saturated ammonium sulphate solution is 70.6%). Optimum enzyme precipitation was between 50% and 55% saturation with ammonium sulphate (Figure 5.13). This is the same range as used by Frieden and Ottesen to purify mushroom polyphenol oxidase in 1959.

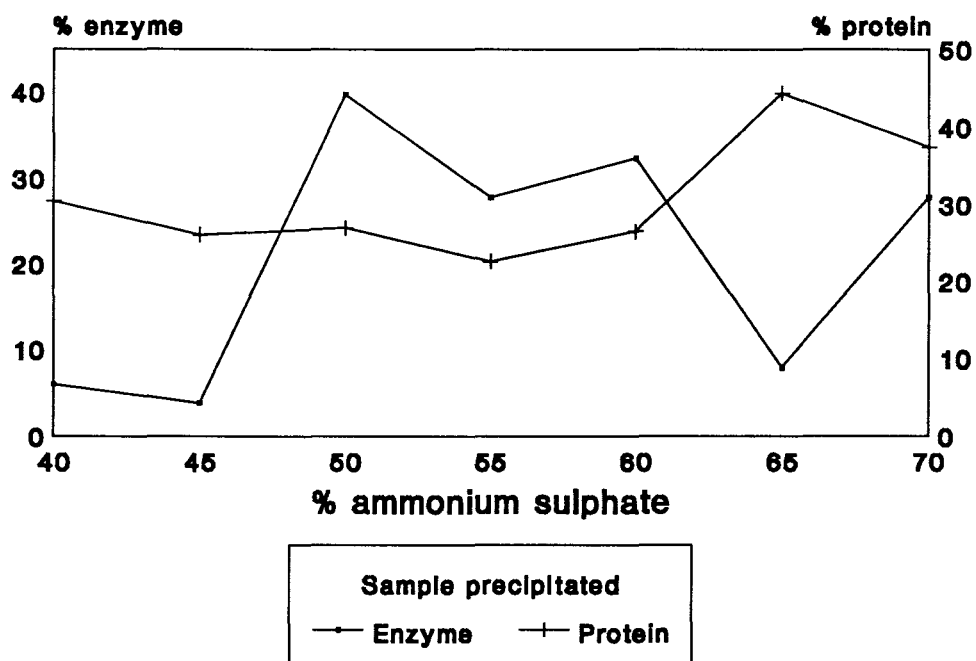


Figure 5.13: Pilot-scale salt fractionation experiment of crude tyrosinase.

Due to the absence of intermediates on product analysis, it was decided to perform the enzyme reaction in an organic solvent so as to retard the reaction rate in an attempt to identify reaction intermediates and elucidate the tyrosinase reaction sequence.

5.2.1.7. Enzyme catalysis in organic media and product analysis

Three experiments were carried out in organic phase using *A. bisporus* crude tyrosinase. Samples were analyzed on UV and GC, using packed Tenax and SE 30 capillary columns for GC analysis.

Figures 5.14 and 5.15 represent the product formation of the aerated enzyme-organic system as analyzed by GC (Tenax column) and UV spectroscopy respectively. A marked colour change was seen through the course of the experiment. After 1.5 hours an immiscible brown precipitate was seen above the chloroform solvent (Plate 5.5). After 3 hours all chloroform had evaporated and only a red-brown precipitate remained. Figure 5.14 shows little phenol and no catechol detected in the solvent up to 1.5 hours. The precipitate (sampled from 3 to 6 hours after the evaporation of the solvent) contained

catechol and high concentrations of phenol. Catechol spiking was performed to confirm the presence of catechol (Figure 5.16). Problems were encountered with the correlation of capillary-GC and UV results. As the maximum absorbances of phenolic compounds on UV analysis were difficult to separate, UV analysis merely provides an indication of the reaction sequence and product formation.



Plate 5.5: The formation of a brown precipitate above the solvent during enzyme catalysis in an aerated organic medium using crude tyrosinase.

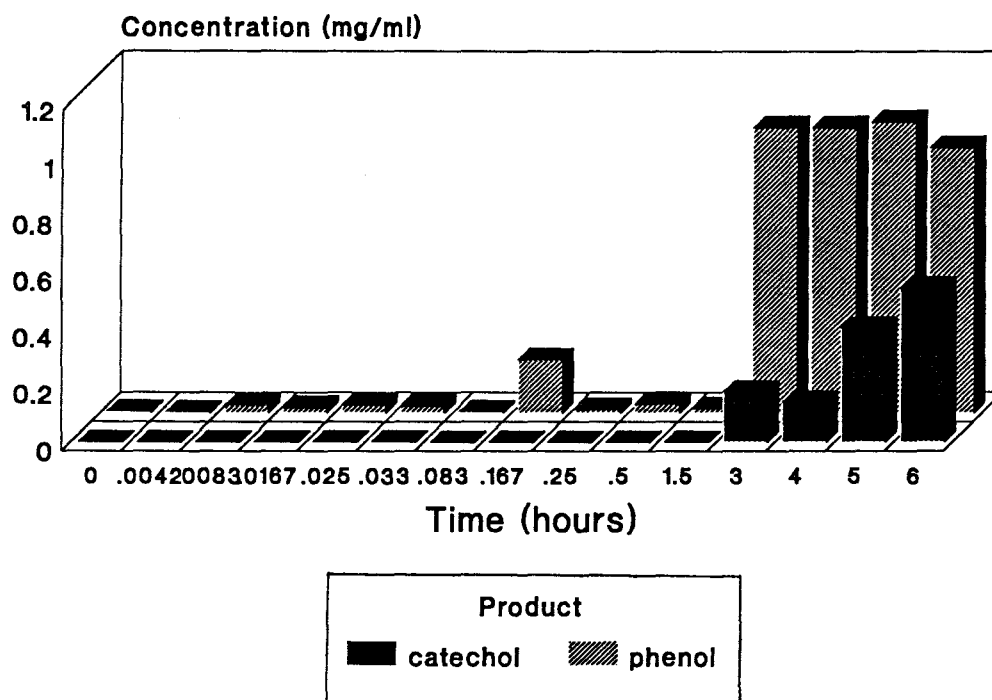


Figure 5.14: Changes in phenol and catechol concentrations as shown by the GC analysis of samples taken during enzyme catalysis an aerated organic medium (14 500 units of crude tyrosinase added to reaction medium).

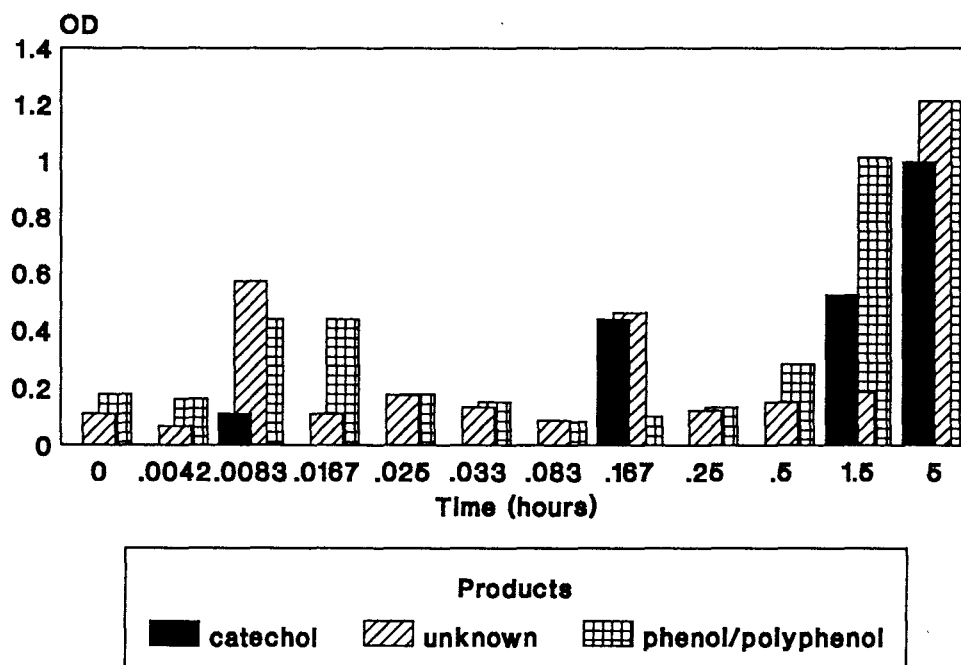


Figure 5.15: The UV analysis of samples taken during an enzyme catalysis in an aerated organic medium (14 500 units of crude tyrosinase added to reaction medium).

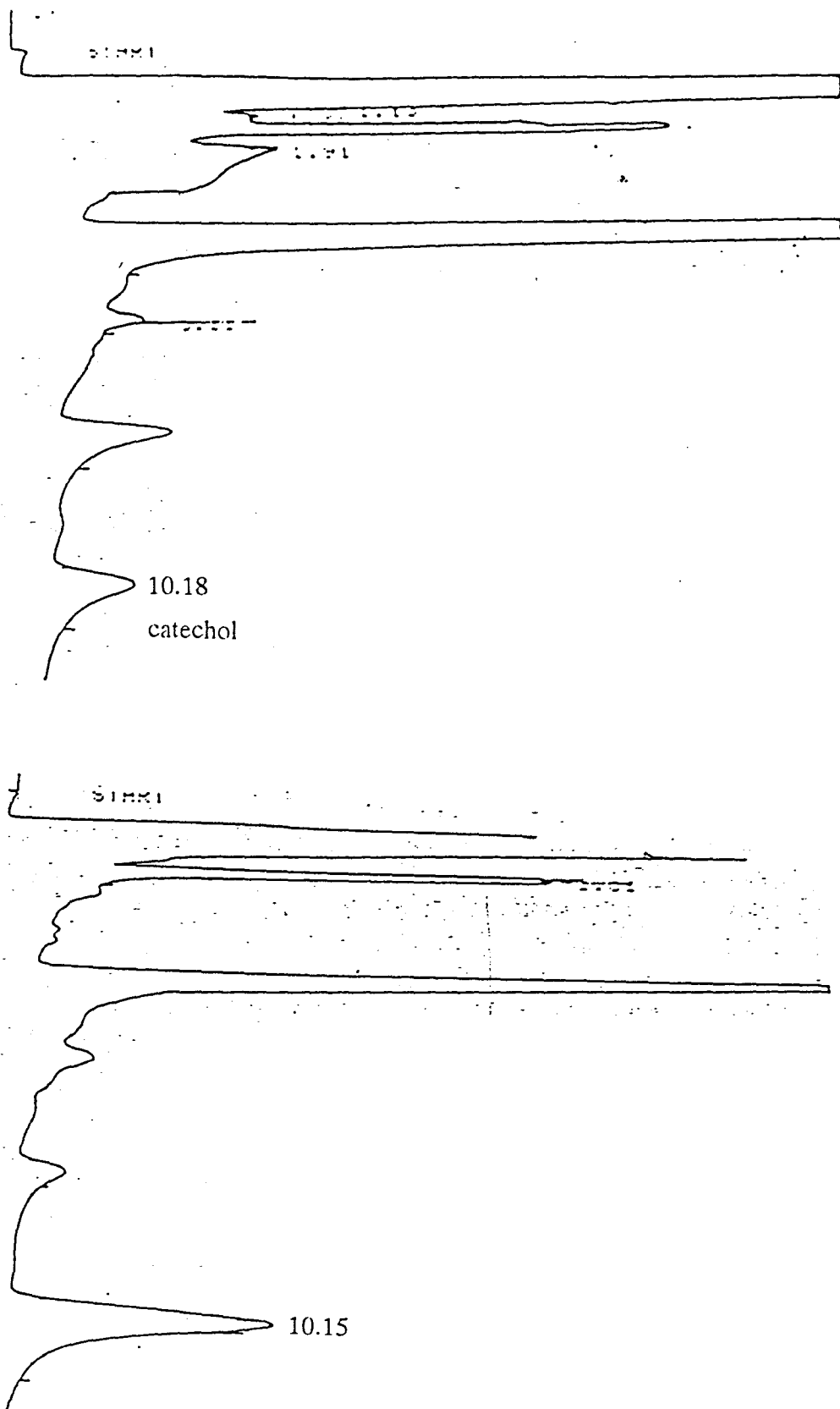


Figure 5.16: A catechol spiking experiment to confirm the presence of catechol.

A: proposed catechol peak - area = 6 816
B: catechol spiking - area = 23 520

Figures 5.17 and 5.18 represent the GC (Tenax column) and UV sample analysis for the unaerated (closed) system of crude tyrosinase organic biocatalysis. A brown precipitate started developing after 30 minutes and was brick-red after 5 hours. The chloroform solvent was colourless. Catechol was detected in the precipitate only, and phenol was detected in both solvent and precipitate.

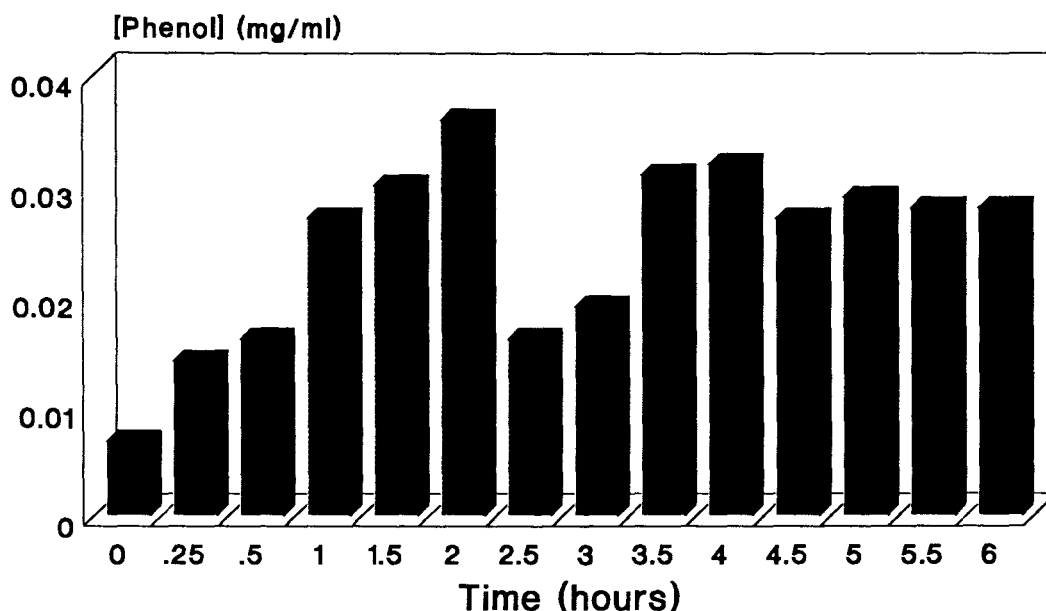


Figure 5.17: Changes in phenol concentration as shown by the GC analysis of samples taken during enzyme catalysis in an unaerated organic medium (14 500 units of crude tyrosinase added to reaction medium).

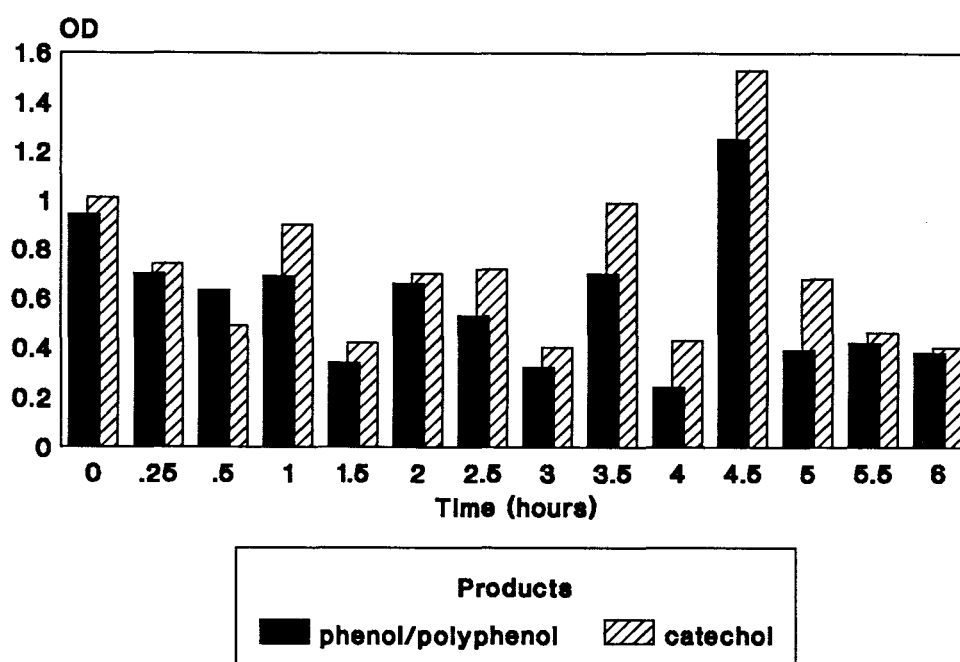


Figure 5.18: The UV analysis of samples taken during enzyme catalysis in an unaerated organic medium (14 500 units of crude tyrosinase added to reaction medium).

A modified aeration experiment was carried out using dried crude tyrosinase. Samples were analyzed by capillary-GC. Phenol was the only reaction intermediate detected (Figure 5.19).

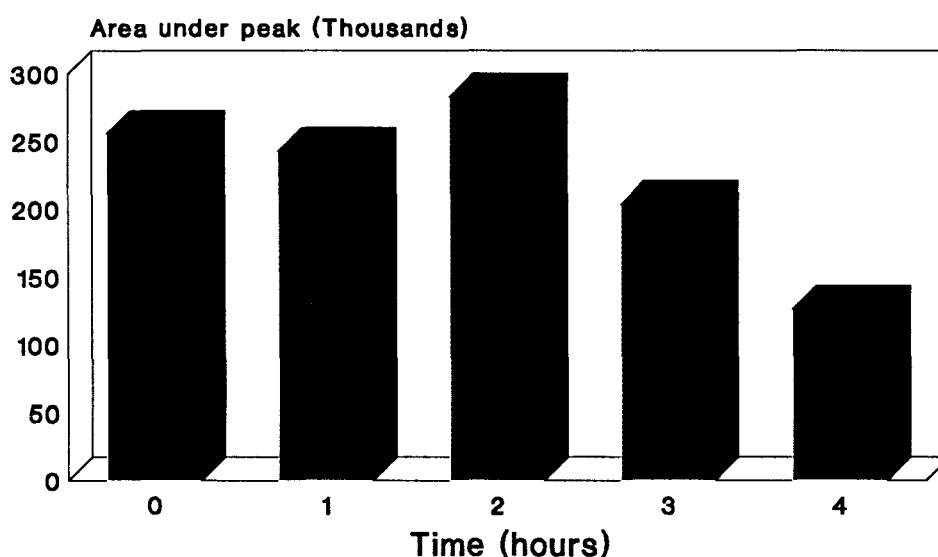


Figure 5.19: Changes in phenol concentration (indicated by area under peak) as shown by the GC analysis of samples taken during a modified organic aeration experiment using 2 755 units of crude lyophilized tyrosinase.

Zymograms developed in organic solvents

In an attempt to determine enzyme activity in organic solvent systems, zymograms were run as before and substrates prepared in chloroform. If successful, the activity of an enzyme in an organic system could be determined visually, eliminating the necessity for complex catalytic experiments in organic media. To test the validity of the system, gels were developed using L-tyrosine and L-DOPA as substrates prepared in chloroform (Plates 5.6 and 5.7 respectively), and compared to aqueous gels. Although tyrosine-developed aqueous and organic gels were identical, DOPA-developed gels were distinctly different. Two bands were seen on gels developed against an aqueous L-DOPA substrate, while only one band was seen in an organic L-DOPA substrate. This result may be ascribed to differences in DOPA solubility in the two solvent systems.

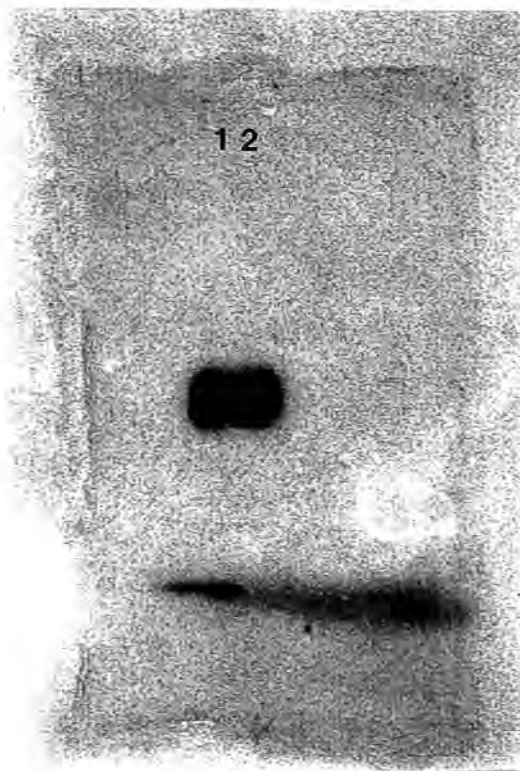


Plate 5.6: A zymogram of crude tyrosinase (lanes 1 and 2) developed using L-tyrosine in chloroform.

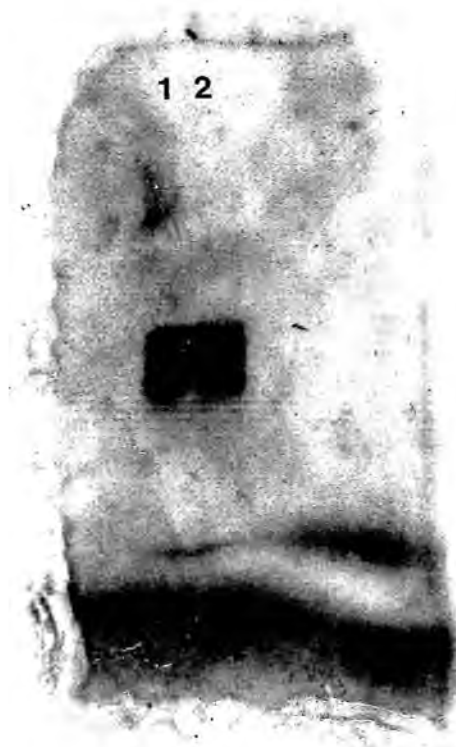


Plate 5.7: A zymogram of crude tyrosinase (lanes 1 and 2) developed using L-DOPA in chloroform.

5.2.2. Crude and commercial *Agaricus bisporus* tyrosinase preparations

Crude and commercial tyrosinase preparations were compared to validate the use of crude tyrosinase as a cheap, but effective enzyme source.

5.2.2.1. Zymogram analysis

Plate 5.8 compares the activity of crude and commercial tyrosinase against L-tyrosine (lanes 4 and 5 respectively). The commercial enzyme appears to have lost the larger subunits on purification.

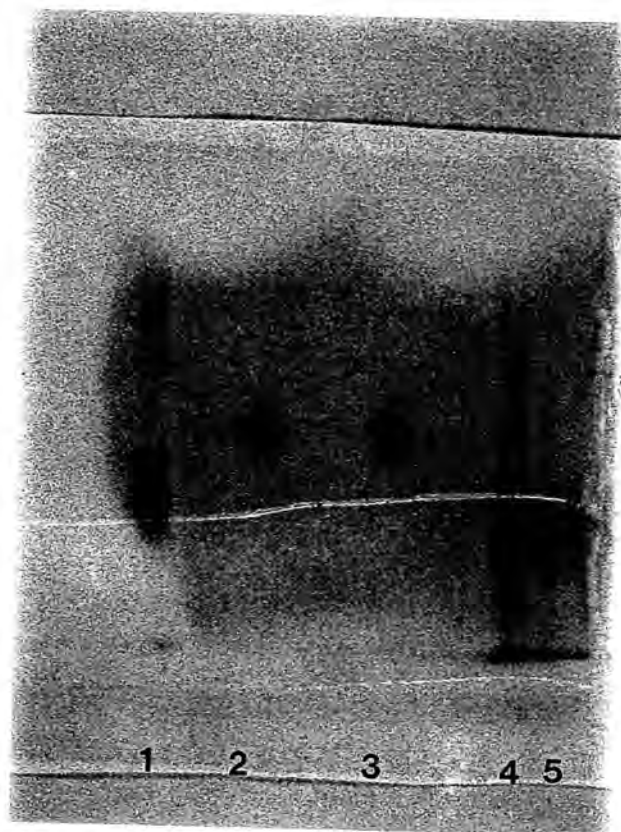


Plate 5.8: A zymogram developed using aqueous L-tyrosine comparing the isozyme pattern of crude (lane 4) and commercial (lane 5) tyrosinase.

5.2.2.2. Phenol removal studies and product analysis

Figure 5.20 is a comparison of the phenol removal efficiencies of crude and commercial tyrosinase as determined by GC analysis using a Tenax column. At this level of activity i.e. approximately 14 500 units, the commercial enzyme removed 86.3% phenol after 1.5 hours and 100% after 5 hours, whilst the crude enzyme removed 69% after 1.5 hours and 73% after 5 hours. These results suggest that the crude enzyme could be used as successfully as the commercial enzyme for the treatment of phenolic effluents. Figure 5.21 represents the UV analysis of samples taken throughout the commercial tyrosinase aeration experiment. Catechol was not detected.

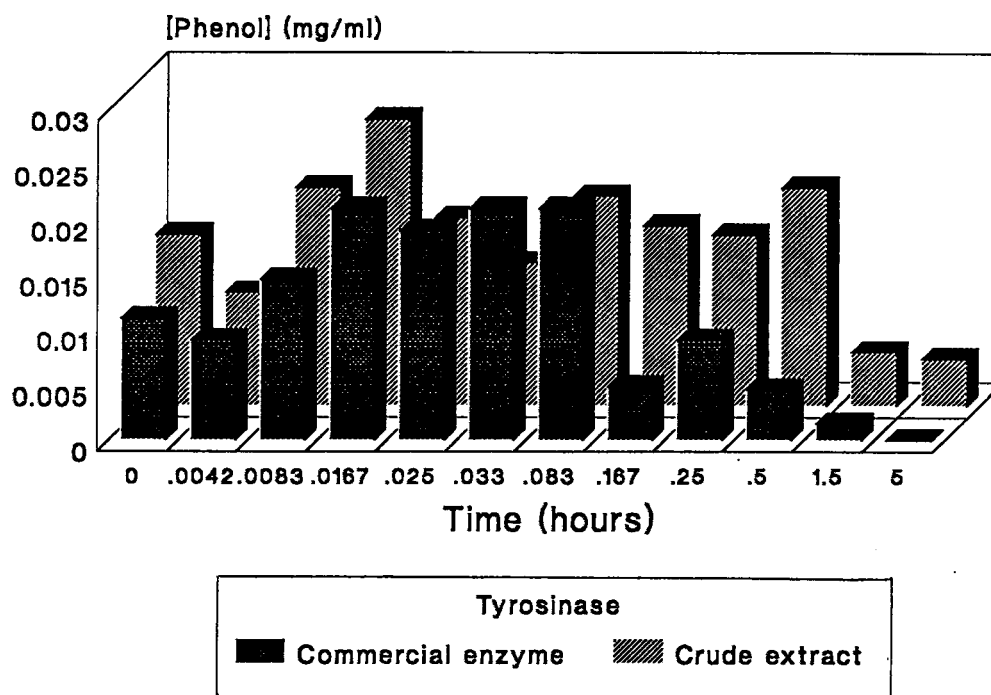


Figure 5.20: A comparison of phenol removal ability of crude and commercial tyrosinase (GC analysis).

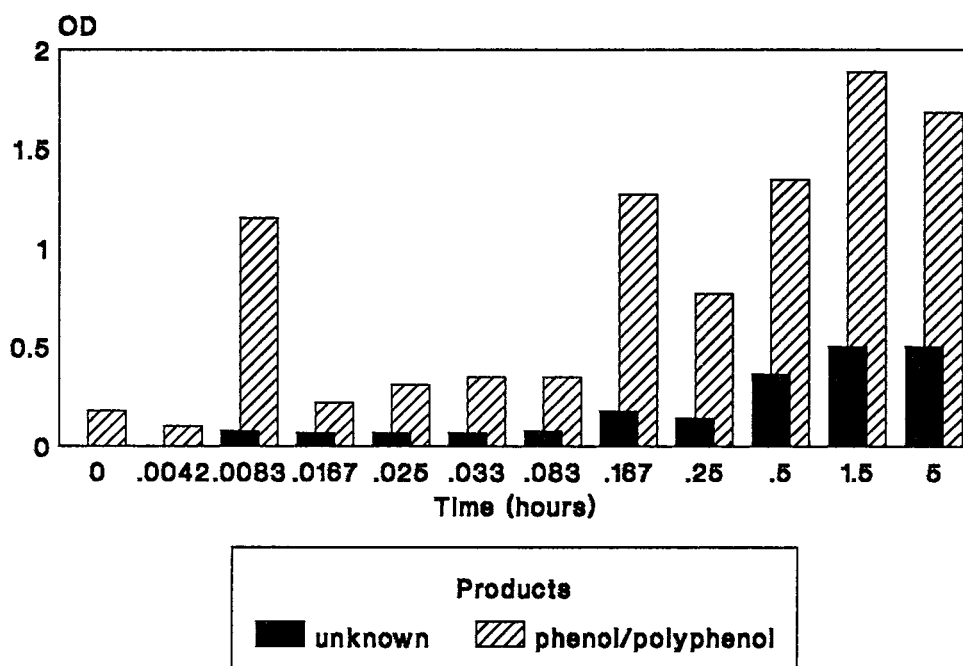


Figure 5.21: The UV analysis of samples taken during an aeration experiment using 14 500 units commercial tyrosinase.

5.2.2.3. Costing

The following result was seen on a cost comparison of crude and commercial mushroom tyrosinase. The price of commercial tyrosinase from Sigma (product no. T-7755) is approximately R70.00 for 25 000 units, while 250g mushrooms (536 units.mg⁻¹ mushroom) costs approximately R4.00. On a units per cent basis, the cost of crude tyrosinase extracted from the mushroom fruiting body is approximately 3.22×10^5 units.cent⁻¹ and commercial tyrosinase 3.57 units.cent⁻¹. Although the commercial enzyme is about 1.3x more efficient than the crude enzyme (Figure 5.20), a comparative costing analysis shows its per unit rate to be 10⁵x higher. It is therefore economically feasible to extract crude enzyme from mushroom fruiting bodies if limited quantities of enzyme are required for specific applications e.g. investigations of enzyme activity in organic media.

5.2.3. Deep-liquid culture of mushroom mycelia

As *A. bisporus* proved to be a good but costly producer of tyrosinase, alternative sources of this particular enzyme were investigated. Although most published reports maintain that tyrosinase is produced solely by the mushroom fruiting body and that the growth of mycelia in liquid culture is directly proportional to the quantity of laccase produced (Wood, 1979), mycelial tyrosinase production has been previously reported e.g. Law (1955) reported the production of tyrosinase by *Lentinus lepideus* mycelia. Tyrosinase production by mycelia was evaluated for two different mycelial sources, *A. bisporus* pure culture, and mushroom spawn.

Shaker flask cultures were labelled as follows:

A's : *A. bisporus* cultivated in Sabouraud broth.

B's : *A. bisporus* cultivated in potato dextrose broth.

C's : Mushroom spawn cultivated in Sabouraud broth.

D's : Mushroom spawn cultivated in potato dextrose broth.

After growth samples were streaked onto 0.01%, 0.1% and 1.0% phenol agar plates to check culture viability. Samples were stained with lactophenol blue and examined

microscopically to check for clamp connections. These would indicate the presence of the heterokaryon tyrosinase-producing stage.

A. bisporus Sabouraud cultures were run for 7 weeks (A1 - A7). All contaminated flasks were discarded. Clamp connections were detected in flasks A2, A3 and A7. Pellets grew abundantly on 0.01% and 0.1% phenol agar plates, turning 1.0% phenol agar plates reddish-brown. Assay results are shown in table 5.5.

A. bisporus potato dextrose cultures were run for 5 weeks (B1 - B5). Contaminated flasks were discarded. Clamp connection formation was seen on staining. Growth on phenol agar was similar to Sabouraud cultures. Assay results are shown in table 5.5.

Mushroom spawn Sabouraud cultures were run for 6 weeks (C1 - C6). Lactophenol blue staining revealed clamp connection formation. Growth was seen on 0.01% and 0.1% phenol agar plates, and pellets turned 1.0% phenol agar slightly brown. Assay results are shown in table 5.5.

Mushroom spawn potato dextrose cultures were run for 5 weeks (D1 - D5). Clamp connection formation was seen in D3 and D4 flasks. Pellets grew abundantly on 0.01% phenol agar, marginally on 0.1% phenol agar and turned 1.0% phenol agar slightly reddish-brown. Assay results are shown in table 5.5.

Table 5.5: Results (units of activity.ml⁻¹) of mushroom mycelial culture extracts assayed against L-tyrosine.

SHAKER FLASK	ENZYME EXTRACT	
	Extracellular	Intracellular
A1	4 640	2 980
A2	4 630	10 820
A3	4 110	4 100
A7	9 710	5 120
B2	3 750	3 710
C1	15 400	15 710
C2	8 080	5 480
C3	3 960	3 390
C4	4 760	4 530
C5	6 780	5 080
C6	7 580	7 880
D4	4 710	5 620
D5	4 210	6 720

The following two extracts were selected for phenol removal studies:

1. *A. bisporus* in Sabouraud broth - A7, extracellular extract.
2. Mushroom spawn in Sabouraud broth - C1, intracellular extract.

Samples taken throughout phenol removal experiments were analyzed on GC using a Tenax column. Good phenol removal efficiencies were recorded. The *A. bisporus* extract showed 75.5% removal and the mushroom spawn 82.9%. No reaction intermediates were detected.

5.2.4. Mushroom compost as a source of tyrosinase

Aqueous and chloroform extractions of mushroom compost did not show any enzyme activity on assay. No phenol was removed from the reaction media during aeration experiments. These results are consistent with most published findings.

5.3. DISCUSSION

5.3.1. *Agaricus bisporus* crude tyrosinase

5.3.1.1. Enzyme extraction

Tyrosinase is a membrane-bound enzyme; both soluble and particulate enzymes have been reported by a number of sources (Bull and Carter, 1973). As the enzyme and phenolic substrate contained within the cell exist side by side in living tissues, maceration or extraction results in enzyme activity and the formation of quinones and other condensation products, eventually causing enzyme inactivation. The use of cold acetone during extraction permits the removal of these interfering phenolic substrates. Although alcohol is a better solvent for the removal of these substrates, acetone prevents excessive enzyme inactivation (Nelson and Mason, 1970).

Once the mushroom paste formed upon filtration had been dried and frozen to break the cells, it becomes necessary to obtain the enzyme in a soluble form. Normally an aqueous solution of moderately high ionic strength and appropriate pH is used for this purpose. Water is used as it is the most convenient and effective solvent. The yield of the soluble enzyme is increased by allowing it to stand overnight (Matthew and Parpia, 1971).

The extraction procedure used in this study proved to be very successful for the isolation of high activity tyrosinase.

5.3.1.2. A comparison of enzyme assay methods

Enzyme assays used were of the batch type i.e. the reaction mixture contained a fixed amount of substrate measured at fixed times. It is important that the reaction is linear over the time period of the assay as the reaction rate drops steadily due to the consumption of substrate. Batch reactions usually depart from linearity when approximately 15% of the substrate has been consumed (Coombs and Hind, 1985). Unfortunately batch assays give an inexact impression of the potential activity of an enzyme, particularly if a low assay

substrate concentration is used and if substrate inhibition takes place. If product inhibition occurs, the assay will overestimate activity (Cheetham, 1986).

Enzyme catalysis has been shown to be an ordered sequential mechanism for both the cresolase and catecholase activities. Oxygen binds first, followed by the phenolic substrate. The formation of the binary complex, EO, may cause a conformational change which creates a more suitable site on the enzyme for the binding of the substrate (Vanni and Gastaldi, 1990).

A number of assay methods were employed for the determination of tyrosinase activity. When selecting a suitable assay system, it is important to define the specificity of the method being tested.

Conflicting information concerning dihydroxyphenolic assay substrates has been reported. L-DOPA and catechol assay methods appear to be unspecific as both laccase and tyrosinase have been detected (Mason, 1966; Phillips and Leonard, 1976; Sykes and Band, 1985). Fling *et al.* (1963) reported the inadequacy of these substrates for *N. crassa* tyrosinase due to very rapid reaction inactivation, whereas Duckworth and Coleman (1970) did not detect any reaction inactivation when assaying for mushroom tyrosinase. Although effective for the assay of mushroom catecholase activity and generally used for the assay of *Streptomyces* tyrosinase, assays against dihydroxyphenolic substrates were not specific enough for the purposes of screening for tyrosinase production.

Monophenolic substrates appear to be more suitable for assaying tyrosinase. However, reports of p-cresol oxidation by laccase have been submitted (Fahraeus and Ljunggren, 1961). p-Cresol was directly oxidized, but rapidly inactivated the laccase enzyme. When added with catechol, both substrates were rapidly oxidized. Catechol therefore seemed to play a protective role.

Tyrosinase was originally named because of its ability to catalyze the oxidation of the amino acid, tyrosine, thus indicating the high degree of specificity of tyrosinase for this substrate. However, a certain degree of irreversible enzyme inactivation does take place during the course of the reaction due to quinone binding (Vanni and Gastaldi, 1990).

Matthew and Parpia (1971) reported tyrosine assays to be misleading when assaying crude enzyme extracts. Despite these obstacles, L-tyrosine remained the most specific substrate for tyrosinase.

Many reports state that the progress curve for L-tyrosine and p-cresol oxidation is characterized by a lag time which can be overcome by adding some diphenol as "activator" (Cabanés *et al.*, 1987; Vanni and Gastaldi, 1990). As tyrosine inhibits its own oxidation (substrate inhibition), it has been suggested that a third binding site exists on the enzyme for the binding of an "activator" (Duckworth and Coleman, 1970). The addition of an activator is generally unnecessary as DOPA is the immediate product of the reaction and is considered an "activator". This catecholase product therefore saturates a specific binding site on the enzyme (Vanni and Gastaldi, 1990). The duration of the lag time depends on the concentration of the phenol substrate, the enzyme source and purity, and the presence of oxidizing and reducing agents (Duckworth and Coleman, 1970). No evidence of this lag period was noted in the present study. The reaction always proceeded very rapidly without any lag or requirement for additional substrates. A similar tyrosinase assay pattern was originally recorded Ashida and Ohnishi in 1967 and again by Scott in 1975.

Jolley *et al.* (1974), cited in Jacobsohn *et al.* (1988), identified oxytyrosinase, a reversible oxygen bound form of the enzyme, as the first intermediate in the oxidation of monophenols to o-dihydroxyphenols and subsequently to quinones. However, three different forms of the enzyme can be distinguished by chemical and spectroscopic studies, i.e. mettyrosinase, deoxytyrosinase and oxytyrosinase (Lerch, 1988). Mettyrosinase is reduced to deoxytyrosinase, molecular oxygen then binds to the binuclear reduced active site of the enzyme (deoxytyrosinase) through an oxidative addition, and produces oxytyrosinase. Tyrosine then binds to the modified active site and is hydroxylated. The product of this step is a complex of the oxidized copper (II) enzyme and DOPA (Vanni and Gastaldi, 1990).

Dopachrome is a red pigment produced during the oxidation of tyrosine. According to Mason (1948), the rate of pigment formation is dependent on enzyme concentration. García-Cánovas *et al.* (1982) reported the accumulation of dopachrome during the lag period of tyrosinase oxidation carried out at pH 7. During this period most of the enzyme

is in the mettyrosinase form and low levels of dopaquinone are produced. However, dopaquinone is unstable at physiological pHs and is rapidly transformed to DOPA and dopachrome. Dopachrome is therefore the first stable product of the reaction (Cabanès *et al.*, 1987).

5.3.1.3. Characteristics of the crude enzyme

Crude mushroom tyrosinase proved to be very stable. After 5 months storage at -20°C enzyme activity was still high when assayed against L-tyrosine (i.e. a determination of the cresolase activity). As the cresolase activity is generally lost before the catecholase activity (Matthew and Parpia, 1971), these results further indicate storage durability. As the commercial value of an enzyme is determined by activity rather than weight of protein, the stability of the enzyme is essential and contributes greatly to its value.

Conflicting reports exist regarding enzyme stability. Scott (1975) stated that an enzyme's stability depends on its level of purity. This stability also depends on the source of the enzyme and its concentration. Highly dilute enzyme solutions may lose a significant amount of activity within 15-20 minutes at 4°C (Scott, 1975). Farkas and Ledingham (1959) reported tyrosinase to be very stable when stored at 4°C for one week. A loss of activity on storage is generally accompanied by the partial oxidation of copper, however experiments have shown that the loss of activity could be ascribed mainly to an alteration of the protein and not to the partial oxidation of the copper.

The constant freezing and thawing of enzyme aliquots during this study did not appear to affect enzyme activity significantly. The crude extract was divided into smaller volumes after extraction in an attempt to prevent unnecessary thawing of large volumes.

Enzyme reactions are highly dependent on pH and temperature. Instant deactivation and denaturation can be expected outside the normal limiting range for each enzyme. The native tyrosinase enzyme exists in a tetrameric form with a compact and globular formation (Khan and Ali, 1986). The three-dimensional enzyme structure essential for catalytic activity is maintained by hydrogen bonding, electrostatic forces, disulphide bridges and hydrophobic interactions so that most of the polar groups are at the surface and most non-

polar groups are situated towards the interior of the molecule. Unsuitable pH and temperature conditions can severely affect the delicate balance of forces necessary to maintain enzyme structure and catalytic activity.

Enzyme-catalyzed reactions increase in rate with a rise in temperature. Unfortunately the rise in temperature also causes a decrease in enzyme stability. The optimum reaction temperature therefore has to be a compromise between these two affects. Enzymes can also be less stable when cooled as the hydrophobic forces maintaining the protein conformation decrease in strength with a decrease in temperature (Cheetham, 1986). In this study, crude tyrosinase was shown to be effective over a wide range of temperatures. This enzyme system therefore possesses the unusual property of being active at both moderately high and comparatively low temperatures.

In general, enzymes are only active over limited pH ranges. Changes in pH are accompanied by changes in the ionized state of the enzyme substrate, and as enzymes possess many ionizable groups, will also affect the binding of the substrate, enzyme conformation and stability and the catalytic activity of the groups in the active site of the enzyme (Cheetham, 1986; Jacobsohn *et al.*, 1988). pH effects may therefore be due to a change in $V_{\max}$ (maximum reaction rate) or K_m (the affinity of the enzyme for the substrate). The optimal pH range for tyrosinase activity is between pH 5 and 7. The enzyme was tested to pH 8 in this study, with no obvious affect on activity.

The substrate specificity of an enzyme originates in its ability to utilize the free energy of binding with the substrate to facilitate the reaction (Klibanov, 1989). In this study, the specificity of enzyme-catalyzed reactions was shown by tyrosinase activity against different enantiomers, with L-tyrosine being favoured. Fling *et al.* (1963) also reported variable activities against L-DOPA, DL-DOPA and D-DOPA. The laevorotatory (L-) enantiomer was oxidized fastest, suggesting a propensity of tyrosinase for this form, and D-DOPA the slowest.

Tyrosinase does not appear to effectively oxidize aromatic diamines or dihydroxyphenols whose functional groups are para- related. As para- oxidation is very complex to achieve chemically, this ability would be of considerable consequence to the chemical industry.

Results of the tyrosinase substrate specificity profile undertaken by this study clearly demonstrates its ability to oxidize both mono- and o-dihydroxyphenols. Enzyme activities were higher against L-tyrosine and p-cresol than phenol due to the influence of side chain orientation and size on enzyme specificity. It has been proposed that a sub-site exists on the enzyme at which rate-determining interactions involving side chain groupings take place (Harrison *et al.*, 1967). Vanni and Gastaldi (1990) also reported the existence of a reaction rate control site. Conflicting reports of dihydroxyphenol oxidation by tyrosinase may be due to the wide variability of tyrosinases from different sources.

5.3.1.4. Enzyme kinetics

Factors affecting enzyme activity include enzyme concentration, substrate concentration and availability, the concentration of cofactors and/or allosteric factors, the presence, concentration and type of inhibitors, pH and temperature. No cofactors are required for tyrosinase-catalyzed oxidation, but determinations of enzyme activity are complicated by substrate and product inhibition. Enzyme activity can be defined in terms of specific activity or kinetic constants and maximum velocities. Tyrosinase is not very appropriate for studies of steady state kinetics as reactions are irreversible and products are either unstable (o-quinones) or are substrates for the enzyme (catechols) (Duckworth and Coleman, 1970). Michaelis-Menten kinetics is used to give an indication of the progression of the enzyme reaction.

Results given in table 5.4 show a high reaction rate ($V_{\max}$) for tyrosinase activity on L-DOPA. The K_m value on L-DOPA corresponds favourably with that of tyrosinase from *Aspergillus nidulans* i.e. 0.25mM (Bull and Carter, 1973). When considering the problems associated with assaying for tyrosinase e.g. substrate and product inhibition, the results in tables 5.3 and 5.4 explain why L-tyrosine is the most suitable assay substrate for determining enzyme activity. The reaction takes place slower on L-tyrosine ($V_{\max}$ values), permitting a more accurate assessment of enzyme activity and a lower substrate concentration is needed to achieve maximum rates (K_m values), thereby reducing substrate inhibition.

Other factors affecting the kinetics of the enzyme have been suggested in the literature. Quinones have a profound effect on the enzyme reaction, either directly by enzyme inactivation and protein precipitation during binding, or indirectly by affecting reaction conditions e.g. redox potential and UV absorption spectra are altered (Coombs and Hind, 1985).

o-Quinones are very labile molecules subject to rapid destruction by reaction with nucleophilic agents such as buffer anions and water. While the quinone is still at the site of formation, a nucleophilic thiol group near the enzyme active site can undergo a Michael-type addition to the quinone molecule (Mason and Peterson, 1965). This association between enzyme and quinone product masks the active site and leads to enzyme inactivation (Vanni and Gastaldi, 1990). Once the quinone diffuses away from the protein surface, the nucleophilic character of the aqueous solvent overshadows the interaction of the enzyme and quinone. The reaction of the solvent with the quinone reduces the quinone concentration so rapidly that the protein-quinone interaction becomes insignificant.

Copper is lost from the enzyme moiety during reaction inactivation. The enzyme nucleophilic group involved in the inactivation reaction is critical to the maintenance of the integrity of the active site. It may either directly bind the enzymatic copper, or maintain a steric structure at the active site which allows strong copper bonding (Mason, 1966).

Reaction inactivation is independent of the rate of oxidation and the initial substrate concentration. High cresolase enzymes are apparently more resistant to reaction inactivation than high catecholase enzymes (Smith and Krueger, 1962). The addition of ascorbic acid for the reduction of quinone has no effect on reaction inactivation as it only reacts with unbound quinone. Extensive reaction inactivation is not seen when 4,5-substituted catechols are used as substrates (Mason, 1966). As the electron-withdrawing ability of the substituents increase, K_m and K_{cat} values decrease and the reaction rate becomes more highly temperature-dependent (Duckworth and Coleman, 1970).

Jacobsohn *et al.* (1988) reported the possible binding of melanin end-products to the enzyme substrate, thereby rendering it unavailable to the enzyme active site. Studies carried out using *S. lividans* (pIJ702) tyrosinase (Chapter 4) indicated the binding of melanin products directly to the enzyme.

5.3.1.5. Protein determination and zymogram analysis

During the isolation of an enzyme it is necessary to monitor its purity by determining the protein concentration of the extract and relating it to activity. This then gives an indication of the specific activity of the enzyme. An efficient protein determination system, e.g. Folin-Lowry or Bradford methods, is essential. Reported protein concentrations of crude tyrosinase extracts were variable, results depend on the extraction method used and the efficiency of extraction. The protein content of crude tyrosinase determined by this study was fairly high, indicating successful enzyme extraction.

Electrophoretic techniques separate charged molecules in an electric field. Convection and diffusion, which would remix protein (enzyme) bands, are reduced by stabilizing the protein on a solid support i.e. polyacrylamide. Zymograms are an application of polyacrylamide gel electrophoresis. Proteins are run under non-denaturing conditions so as to maintain enzyme activity. The relative migration of proteins on non-denaturing gels is therefore a function of molecular mass and charge (Williams and Wilson, 1981). During this study gels were run at 4°C to minimise heat denaturation. As zymograms were investigated as enzyme detection systems, mini-gels were set up so as to ensure speedy analysis rather than detailed resolution.

Plates 5.1, 5.3 and 5.4 (developed against aqueous L-tyrosine, phenol and p-cresol respectively) show a diffuse band at the same position on all gels. This band ran slightly further on the gel developed against phenol. Due to the size, resolution and appearance of the band, it appears as if more than one isozyme is present. Jolley and Mason (1965) reported that an equilibrium of 5-6 different tyrosinase isozymes may exist at any one time. This was substantiated by Jolley *et al.* (1969) who stated that more than one band is often present in sieving acrylamide gels. It therefore seems probable that the large enzyme band is heterogeneous.

As this broad band appeared when developed against monophenols and the o-dihydroxyphenolic substrate, L-DOPA, the isozyme at this position must have both cresolase and catecholase activities. This band corresponds with one previously identified to be a tetrameric tyrosinase isozyme (Jolley *et al.*, 1969). Jolley *et al.* (1969) reported that isozymes seen on acrylamide and starch gels are composed of an equilibrium mixture of polymeric forms with the tetramer predominating. The isozyme(s) at band A on plate 5.2 may therefore be a higher polymeric form and larger enzyme structure than the tetramer (band B) as it has migrated slower. As band A only appeared on development against DOPA, it probably has mostly catecholase activity. The isolation of isoenzymes of different catecholase and cresolase activities have been reported by a number of researchers.

The presence of the second enzyme band on plate 5.2 may be due to the solubility of L-DOPA in the reaction medium. As DOPA is the most soluble of all substrates tested, its solubility may have ensured interaction with a particular isozyme, resulting in the formation of band A. However, developing gels against water-insoluble substrates e.g. methoxyphenol and p-tertiary-butylphenol, show that the solubility of a substrate does not determine its activity with the enzyme (Burton, pers. comm.). The enzyme-substrate reaction appears to take place at the gel interface. The solubility of the substrate may however affect the extent of the reaction, which would explain the poor results of phenol and p-cresol zymograms. Phenol and p-cresol results of this study confirm results reported by Leonowicz and Trojanowski in 1978. Although L-tyrosine is fairly insoluble in aqueous media, it is also the most specific substrate for the enzyme, ensuring good activity against tyrosinase.

The presence and size of substrate side-chains also appear to affect the extent of the reaction. The degree of enzyme-substrate interaction or colour formation seems to increase from phenol to p-cresol, to tyrosine and finally to L-DOPA. This is consistent with the suggestion that tyrosinase has a stereospecific reaction rate control site. Substrate side chains bind to the control subsite by chelating with copper and function as reaction rate modulators (Harrison *et al.*, 1967). An increase in side-chain size therefore has a favourable impact on the enzyme reaction.

All enzyme fractions may therefore possess the same peptide sequences for activity, but the conformational state and state of aggregation may differ, resulting in the formation of several isoenzymes.

5.3.1.6. Phenol removal studies and product analysis

As mentioned in section 5.2.1.6, phenol oxidation can be associated with rapid and distinct colour changes. The immediate products of the oxidation reaction i.e. catechol and quinone, are colourless (Bonner, 1957), but the polymerization products are coloured. The black precipitate seen at the end of the reaction is the polyphenolic polymerization product which is used in the manufacture of a variety of industrial products. In addition to its use in the clarification of industrial effluents, this enzyme system can therefore also be applied to the production of commercially valuable polyphenolic compounds.

Polyphenols are useful as starting materials and curing agents in the synthesis of heat-resistant epoxy resins. They can be used for the manufacture of heat-resistant, chemically resistant and radiation resistant materials, and also as antistatic materials for coating and other applications. Polyphenols can be produced by a number of methods e.g. oxidative heteropolycondensation in the presence of a catalyst(s). Although useful, this technique is not economically viable. A successful method for preparing polyhydroquinone, used as a binder for carbon and organic plastics such as graphite-containing grease, has been described. Unfortunately the H_2O_2 necessary for production is costly, has an explosive nature and requires costly corrosion-proof processing equipment. A patent was issued to Ragimov *et al.* (1983) for the successful production of polyphenols by a number of methods using oxygen as oxidant e.g. using phenol, alkyl phenol and cresol as starting materials.

Although the chemical production of polyphenols is highly effective, there are a number of advantages to using enzyme systems for the preparation of this compound (Ragimov *et al.*, 1983):

1. Enzymes use an easily available and unaggressive oxidizer and provide a low-cost process for preparing polyphenols;

2. The enzyme-catalyzed process is safe;
3. The enzyme-catalyzed method makes possible the most complete use of auxiliary materials;
4. Pollution of the environment by reaction products is avoided;
5. Unreacted monomers of the phenolic series are easily separable and reusable.

The oxidation of phenolic compounds by the tyrosinase enzyme can therefore be harnessed for the successful production of polyphenolic compounds.

Removal studies showed that phenol concentration and precipitate formation are inversely related as the phenolic substrate is continuously being converted to the polyphenolic product. There is a distinct fluctuation in the phenol concentration of stored samples (Figure 5.6). This pattern is typical of similar patterns seen in aeration experiments and enzyme assays e.g. Figure 5.22.

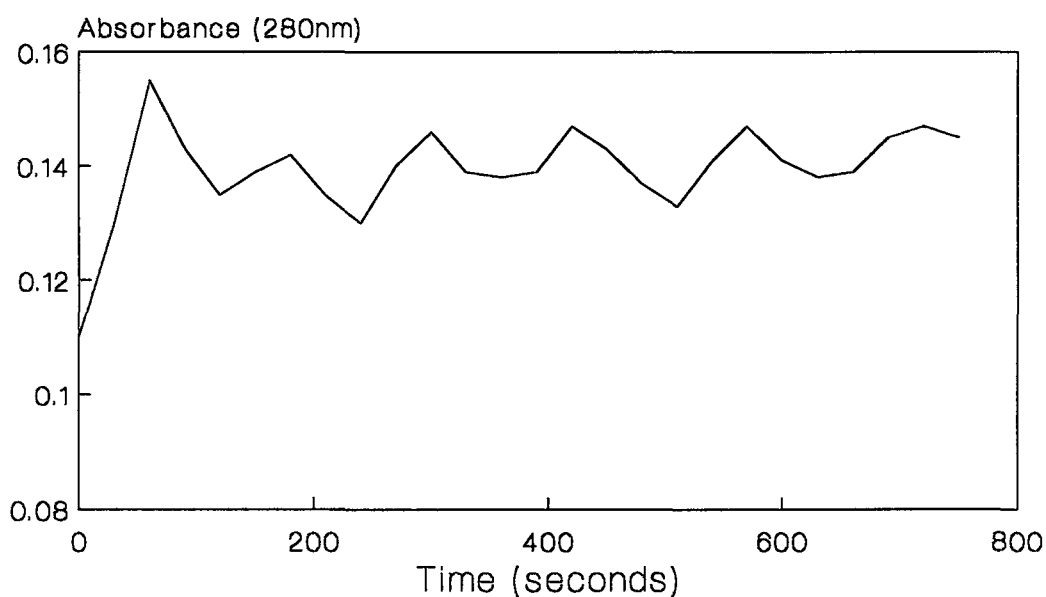


Figure 5.22: An example of fluctuating product patterns detected on assay.

It appears as if a binding-release system is taking place. The substrate e.g. phenol, is added to the enzyme, undergoes the reaction and the product is removed to free the enzyme for the next reaction. Two products may result from this reaction, i.e. catechol and

quinone. Unreacted phenol may also be released, accounting for the unusual surges in phenol concentration.

Two substrates are also available to the enzyme i.e. catechol, the product of the first reaction, and phenol, which is still available as substrate in the reaction medium. The product of the first reaction therefore becomes the substrate for the second reaction. Hence substrates and products are bound and released throughout the reaction - a proposed system is depicted in figure 5.23.

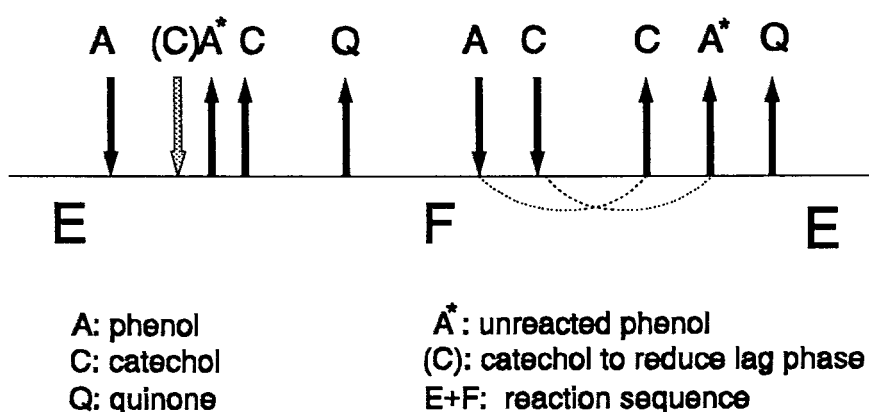


Figure 5.23: A proposed binding-release mechanism for tyrosinase in aqueous media.

This enzyme system appears therefore to be much more complex than the traditional one substrate, one product enzyme reaction, as it becomes necessary to define Michaelis-Menten, inhibition and equilibrium constants for each reactant in the mechanism.

It remains to be determined whether the enzyme catalyzes the reactions sequentially, or whether different substrate-product conversions are carried out at different points by different enzyme subunits. More research on subunit enzymology is required before an attempt at answering this question can be made.

This binding-release mechanism is substantiated by the UV analysis of samples (Figures 5.7 and 5.8). Phenol substrate and polyphenolic product peaks were grouped

together as they could not be reliably identified and separated on UV analysis. An increase in phenol peak size could therefore indicate the formation of the polyphenolic product.

Conclusions drawn from results of phenol removal studies are in direct conflict with the proposed stoichiometry of tyrosinase-catalyzed oxidation reaction. If unreacted phenol is being released from the enzyme site, the reaction cannot be completely stoichiometric.

Fluctuations in phenol concentration are more perceptible in samples stored at -20°C before analysis than in samples analyzed immediately upon completion of the experiment. It is possible that some evaporation of reaction medium takes place over time, thereby concentrating the phenol and amplifying the discrepancy between the two sets of samples.

It is obvious from figure 5.8 that a number of changes occur upon storage e.g. an unknown product is detected in all stored samples, but only in the last three of the samples immediately analyzed. This product is assumed to be an auxiliary oxidation product of the aqueous enzyme reaction and may be a polyphenolic product. Quinone is detected throughout the reaction, which is expected if the proposed binding-release mechanism is taking place. Samples stored at -20°C again deviate; presumably the composition of products in the media changes upon storage, probably due to further oxidation.

Figures 5.7 and 5.10 show that the amount of enzyme added to the reaction medium contributes to the detection of products upon analysis. Although it is well known that enzyme concentration affects the reaction, an attempt was made to verify UV spectroscopic results and identify reaction intermediates by GC-mass spectroscopy (GC-MS). Unfortunately the range of products and intermediates produced by this reaction could not be successfully identified. It was thought that many of the products were transient, probably being precursors of the final insoluble polyphenolic material. Their identification was therefore not pursued.

Phenol removal studies showed that the efficient aeration of reaction medium is critical to the successful and complete oxidation of phenolic substrates. Vigorous agitation is necessary as tyrosinase has a low affinity for oxygen and a high affinity for its phenolic

substrate (Bull and Carter, 1973). Product formation is also suppressed by oxygen limitation (Shivprasad and Page, 1989).

The phenol removal efficiency of a particular system therefore depends on the rate of oxidation and enzyme and substrate concentrations (Atlow *et al.*, 1984). The pH of the reaction medium did not appear to have much effect on the phenol removal efficiency of crude tyrosinase, only the pattern of product development e.g. little of the unidentified product was produced at pH 6.

The above results confirmed both the complexity and the potential value of this enzyme system. Many intricacies need to be clarified before tyrosinase can be utilized to its full potential for the treatment of aqueous phenolic wastewaters.

Enzyme-product binding

A proposed explanation for the absence of reaction intermediates on GC analysis, was enzyme-product binding. A protein-dissociation experiment was carried out to check for catechol-enzyme binding, but results were negative. This finding was substantiated by Scott (1975) who claimed that quinone is more likely to bind or mask tyrosinase than catechol. However, a second experiment checking for quinone-enzyme binding was also negative. As catechol and quinone were not detected by GC, oxidation of the catechol and polymerization of the quinone had probably already taken place.

According to Doddema (1988), 70% quinones can be detected in water at pH 6. At higher pH values, or when maintained at pH 6 for longer than 1 hour, all quinones disappear due to polymerization. It is therefore interesting that quinones were not detected by GC, either at pH 6 or pH 8. It is possible that quinone and catechol concentrations in the reaction media were too low for detection. However, 0.05 mg.ml⁻¹ quinone and 0.5 mg.ml⁻¹ catechol standards were detected on capillary-GC. A more plausible suggestion is that intermediates are not present in the media long enough to be detected. The enzyme reaction takes place very quickly. This proposal does not, however, explain the detection of intermediates by UV spectroscopy. An unsuccessful attempt was made to verify UV results by GC-MS.

5.3.1.7. Enzyme catalysis in organic media and product analysis

Catalysis in organic solvent systems were performed in an attempt to detect reaction intermediates and verify the progression of the tyrosinase reaction.

Conducting enzyme reactions in organic solvents resolve many of the problems normally associated with aqueous media e.g. water-dependent side reactions such as the polymerization of quinones, the insolubility of substrates and unfavourable thermodynamic equilibria. Reaction inactivation is greatly reduced in organic solvents and a third of the enzyme activity is retained after catalysis (Kazandjian and Klibanov, 1985). As chloroform is less polar than water, the enzyme is not inactivated (Dordick, 1989). Substrate specificities and the rate of reactions may be similar in aqueous and organic systems (Kazandjian and Klibanov, 1985) e.g. the rate of hydroxylation of acetyl-N-D,L-tyrosine ethyl ester (ATEE) (Doddema, 1988), but organic reactions are generally slower than aqueous reactions. Enzyme inhibition by substrate concentration is also reduced due to the nature of the organic solvent. This lowers the adverse effect of the substrate and leads to higher reaction rates (Antonini *et al.*, 1981). A small volume of water is added to the reaction medium to maintain the catalytically active conformation of the enzyme. The effect the solvent has on the reaction is due to its interaction with the enzyme-bound water layer, rather than the enzyme itself. Enzyme hydration is essential as it relaxes the enzyme structure and therefore aids enzyme activity (Zaks and Klibanov, 1988).

Before using an enzyme in an organic system its structural and stereochemical specificities need to be well documented, so as to confidently predict the structure and configuration of the products. When used industrially it is important for the enzyme to be functionally pure, stable, cheap, easy to use and commercially available (Wiseman, 1986).

The most striking result seen during this study was the detection of catechol as an intermediate of the enzyme reaction. The presence of this intermediate was confirmed by a catechol spiking experiment. Although o-quinones have been reported to be stable for several hours in chloroform (Doddema, 1988), no quinone was detected. The addition of a reductant, e.g. ascorbic acid, for the conversion of quinone back into catechol was therefore not necessary. This was fortuitous as the use of a reductant generally leads to

enzyme inhibition (Doddema, 1988). The development of a brown polyphenolic precipitate above the chloroform (Plate 5.5) indicated that the reaction had run to completion, suggesting that quinone probably was present, but in a polymerized form undetected by GC.

In an organic system quinones normally partition into the bulk solvent. The system in this study contained a higher concentration of aqueous media than was necessary for enzyme hydration; polymerized quinone may therefore be contained within the aqueous layer. However, a modified aeration experiment containing minimum aqueous medium also did not show any intermediates on analysis. It is possible that an undetectable amount of quinone was present in the chloroform solvent. A study carried out by Doddema in 1988 also reported an absence of quinones in both water and organic solvents. He suggested that the quinones polymerized as rapidly as they were produced to a chloroform-soluble, non-reducible compound. Quinones are therefore very unstable products, particularly in an aqueous medium.

The partitioning of enzyme, substrate and products due to solubility was evident in this study. The aqueous enzyme extract immediately formed a layer above the chloroform solvent upon addition to the reaction medium. This organic system contained approximately 0.9% water, somewhat higher than the 0.5% requirement for tyrosinase in organic media (Dordick, 1989). Enzyme activity increases with an increase in solvent water content up to 1.0% (v/v) (Martinek *et al.*, 1981). At this solubility limit enzymes tend to form glue-like fibres devoid of enzyme activity (Zaks and Klivanov, 1988). The product yield of this system may therefore have been maximized by the removal of some water from the system. To improve product yield it is also necessary to reduce the thermodynamic activity of water (a_w) significantly below 1 i.e. the value for pure water and any water-saturated organic liquid. A_w is equivalent to the relative humidity of the atmosphere in equilibrium with the solution (Pirt, 1975). Lowering the water activity prevents enzyme unfolding (O'Fagain *et al.*, 1988). The critical water content of this system should be maintained close to its optimal value (Khan *et al.*, 1990). A water-immiscible solvent such as chloroform also supports enzyme activity in the presence of low levels of aqueous buffers e.g. 0.25% v/v (Dordick, 1989).

Two-phase systems are generally established when immiscible solvents are employed. The water contains the enzyme and the organic solvent the substrate. In this study, the phenolic substrate was, however, detected in both the chloroform solvent and aqueous enzyme layer. On shaking or stirring, a transfer of substrate normally takes place from the organic to the water phase. Catalysis therefore only takes place in the water phase (Antonini *et al.*, 1981). Shaking is controlled as the enzyme can become destabilized, and is dependent on the nature of the solvent and substrate and on the amount of enzyme used. The reaction rate is dependent on the concentration of the substrate in the water phase rather than on its total concentration in the mixture. Once the substrate has undergone the enzyme-catalyzed reaction, products are formed which eventually return to the organic phase. In the present study, catechol was fairly concentrated in the aqueous layer and did not appear to return to the organic phase, probably due to its insolubility in chloroform (Weast and Astle, 1980). The brown colour of the precipitate indicated the presence of the polyphenolic product (Figure 5.14), i.e. a water-dependent product. The enzyme and polyphenolic product therefore exist together in the aqueous layer, complicating the removal of both enzyme and product. The differential solubility of both substrate and product limits product conversion and could be successfully resolved by immobilizing the enzyme. Immobilization would also eliminate intraparticle diffusional limitations (Kazandjian and Klibanov, 1985).

Although chloroform holds 10x more dissolved oxygen than aqueous media, additional aeration (in the aerated reaction system) speeded up the enzyme reaction. Klibanov *et al.* (1981) reported that using oxygen instead of air would further enhance the speed of the reaction. The concentration of catechol in the precipitate of the aerated system (Figure 5.14) was substantially higher than that of the non-aerated system (Figure 5.17). The aerated system also showed more extensive polyphenolic production.

No intermediates were detected during the modified aeration experiment using dried tyrosinase (Figure 5.19), but 50% phenol was removed. As there is no selective force in the organic solvent to change the ionization states of charged protein groups of the enzyme, the chemical state of the lyophilized enzyme would remain unchanged from that of the aqueous solution from which the enzyme was last recovered (Dordick, 1989). It is

therefore important that the aqueous enzyme solution be maintained at the optimum pH before lyophilisation is carried out.

As stated in section 5.3.1.6, it was not possible to reliably separate the phenolic substrate and polyphenolic product peaks on analysis. Separating and correctly identifying UV peaks was also a demanding task and two unidentifiable peaks were detected. Klibanov *et al.* (1981) reported that carrying the reaction out at 0°C reduced the formation of auxiliary hydroxylation products. As all the experiments of this study were carried out at room temperature, this may explain the detection of unidentifiable products.

The choice of solvent for enzyme reactions in organic media is critical. The nature of the solvent can affect enzymic catalysis in many ways (see Chapter 1). Chloroform is a suitable solvent as it is immiscible and polyphenol oxidases are stable in this solvent (Antonini *et al.*, 1981). Hydrophobic solvents are generally more suitable than hydrophilic ones as they have little affinity for water, which therefore remains bound to the enzyme. Chloroform is also a valuable solvent as it can positively interact with diffusible products of the enzyme reaction e.g. it reduces the catalytic activity of peroxidase-catalyzed phenol oxidations as it is a good phenoxy radical quencher (Dordick, 1989).

The range of compatible solvents can be increased by binding the tyrosinase biocatalyst to relatively hydrophilic supports e.g. tyrosinase is more stable when precipitated onto glass powder than in free, suspended form (Laane *et al.*, 1987). This support helps to stabilize the essential water layer around the enzyme. Enzymes are also less susceptible to solvents when immobilized e.g. D'Angiuro *et al.* (1987) reported that free horse radish peroxidase (HRP) is very sensitive to solvents used even at low concentrations.

Catalytic studies in organic media were successful in that a reaction intermediate of the tyrosinase enzyme reaction (i.e. catechol) was detected. Due to the range of products that can be produced by these systems, organic biocatalysis is a promising area of enzyme research and one which requires extensive investigation.

Zymograms developed in organic solvents

Chloroform-developed zymograms were investigated for the rapid detection of organic tyrosinase reactions. Results were compared to aqueous zymograms to determine similarities between the two systems. This technique can only be definitive of an organic reaction if results are unambiguous.

No shrinkage was seen when a slab of 7.5% polyacrylamide (80% (w/v) water, 20% polymer) was placed into chloroform, indicating that either the solvent was directly and completely replacing the water inside the gel, or that no movement of water or solvent was taking place. Due to the extreme immiscibility of chloroform, the latter suggestion appears to be more feasible. The polyacrylamide itself does not affect the reaction as it does not preferentially extract water or chloroform (Woerdenbag *et al.*, 1990) and has a low solubility in chloroform. The system and composition of the gels is designed such that the polyacrylamide support will have little effect on the enzyme reaction. However, if the chloroform was replacing the water held within the gel, some change in gel size or structure would be expected. When 20%, 50% and 100% ethanol and acetone were tested as organic solvents, severe gel shrinkage was seen. These solvents are inappropriate for tyrosinase-catalyzed conversion reactions due to their hydrophilic nature which encourages enzyme unfolding and inactivation.

Assuming that no water-chloroform replacement takes place, the concentration of water within the system is far higher than the 0.5% requirement for tyrosinase activity in organic systems. The A_w has not been reduced below 1, indicating that the system cannot be classified as a true organic reaction. Plates 5.1 and 5.7 i.e. zymograms developed against aqueous tyrosine and tyrosine in chloroform respectively, were identical. Plates 5.2 and 5.8 i.e. zymograms developed against aqueous DOPA and organic DOPA respectively, were distinctly different. This variation could be attributed to the difference in solubility of DOPA in water and chloroform, possibly affecting substrate reactivity with particular isozymes. As DOPA reacts in chloroform despite its insolubility, the reaction must be taking place at the enzyme/substrate interface. Substrate solubility therefore determines the extent of a reaction.

Isoenzymes are defined by Abercrombie *et al.* (1973) as variants of a given enzyme occurring within a single organism. They have the same substrate specificity and catalyze the same reactions, but have slight differences in molecular structure, making identification and separation possible. They may also differ quantitatively in activity. It is therefore feasible that different isoenzymes may have different solvent specificities e.g. the chloroform solvent may exclude the high catecholase isozyme fraction by altering its structure. As isoenzymes are influenced by pH and ionic conditions, the solvent may play a significant role in determining isoenzyme formation.

Although the DOPA/chloroform zymogram was distinctive and reproducible, other substrate gels reacted as in aqueous systems. It is therefore premature to promote this method for the identification of organic enzyme reactions, as it is not clear whether the DOPA result was due to the influence of the solvent or due to substrate solubility. Although organic zymograms represent a rapid and efficient tool for the determination of organic tyrosinase reactions, it is not practical to employ this technique, particularly due to the difficulties associated with creating a truly monophasic organic electrophoretic system.

5.3.2. Crude and commercial *Agaricus bisporus* tyrosinase preparations

Crude and commercial tyrosinase preparations were compared on the basis of zymogram analyses and phenol removal studies. A costing analysis stressed the advantage of using crude tyrosinase for experimental studies. Although possibly financially unsuitable for extensive aqueous applications, the crude enzyme appears to be suited to large-scale organic biocatalytic reactions as less enzyme is required for these conversions.

Zymograms show a distinct difference between crude and commercial enzyme preparations i.e. Plate 5.9, a zymogram developed against L-DOPA. The minor band of lane 4 (crude tyrosinase) is not present in lane 5 (commercial tyrosinase), indicating the loss of an isoenzyme(s). Enzyme specificity therefore appears to change during purification. This result was supported by Scott (1975). Kumar and Flurkey (1991) reported significant variations of enzyme activity and isoenzyme forms between different commercial enzyme preparations.

Phenol removal studies showed that commercial tyrosinase has a better dephenolizing ability than the crude enzyme preparation. Although purification of a crude enzyme normally reduces its activity (Matthew and Parpia, 1971), concentrating (drying) the enzyme induces the association of subunits, thereby strengthening the enzyme (Jolley *et al.*, 1969) and increasing its specific activity.

Figure 5.20 shows the same fluctuating pattern of phenol concentration for both crude and commercial tyrosinase. This pattern is a further illustration of the binding-release system described in section 5.3.1.6. Unlike the analysis of crude enzyme samples (Figure 5.10), UV analysis of commercial tyrosinase reaction medium (Figure 5.21) showed no intermediates. This variation appears to be linked to changes in enzyme activity brought on by purification. It has been widely reported that the cresolase activity of the enzyme diminishes rapidly during purification as it is fairly unstable (Scott, 1975). Catechol and quinone levels in the media may therefore be too low to detect. Despite the lack of intermediate detection by GC analysis, commercial tyrosinase demonstrated a very competent phenol removal efficiency.

5.3.3. Deep-liquid culture of mushroom mycelia

Despite many reports of tyrosinase production by mushroom fruiting bodies exclusively, laccase and tyrosinase presence in the same mycelium was reported by Käärik as early as 1965. The detection of tyrosinase in mushroom mycelium is therefore possible, although unusual. As a proportion of total tyrosinase activity has been ascribed to laccase production (Matsubara and Iwasaki, 1972), extracts were assayed against tyrosine to correctly identify the enzyme. The dark-brown colouration seen on 1.0% phenol agar plates confirmed the presence of tyrosinase. Results of both *A. bisporus* and mushroom spawn tyrosinase aeration experiments further verified the identity of the enzyme and demonstrated its remarkable phenol removal ability.

Intra- and extracellular enzyme activities against L-tyrosine were high and a good correlation was seen between the formation of clamp connections and enzyme activity. Although good enzyme production was seen in both growth media, Sabouraud broth seemed more suited to tyrosinase production as potato dextrose broth was prone to

contamination. The readily available organic compounds provided by the inclusion of horse-manure broth into growth media enhanced mycelial growth and as cell growth and enzyme production appear to be closely linked, aided enzyme production. Manure has been used as a substrate for single cell protein production by *Pseudomonas fluorescens* and *Rhodopseudomonas* sp. for many years (Litchfield, 1985). The importance of adequate growth substrates for enzyme production has become very evident throughout this study. The inclusion of horse-manure extract therefore provided the essential nutrients needed to enhance cell production in this particular system.

The phenol removal efficiency shown by both *A. bisporus* and mushroom spawn tyrosinase was very good and compared favourably with that of the crude and commercial fruiting body tyrosinase enzymes. This result is highly significant as efficient phenol removal by a mycelial tyrosinase has not been noted in the literature covered throughout this study. The production of mushroom tyrosinase by mycelia presents a new aspect of commercial enzyme production i.e. enzyme production in deep liquid culture. The production of tyrosinase in a liquid culture system is both labour- and space-saving. The enzyme yield is high and is produced in a form which is easy to isolate. Extracellular enzyme can be harvested by simple filtration techniques, whereas intracellular enzyme can be collected by harvesting the biomass. Once the enzyme has been extracted, the biomass can be dried and used as a mushroom flavourant. Problems encountered with harvesting and enzyme extraction would be negligible in comparison with economic advantages gained by a fermentative enzyme production process. The production of tyrosinase in deep-liquid culture is therefore a very valuable alternative to the large-scale production of mushroom fruiting bodies as a source of tyrosinase.

5.4. CONCLUSIONS

A number of conclusions can be drawn from the results obtained in this study:

1. Although the tyrosine assay method is not ideal for assaying crude enzyme extracts, it is the most specific method of determining tyrosinase activity.
2. Tyrosinase is stable when stored at -20°C.
3. Tyrosinase is effective over a wide temperature and pH range.

4. Tyrosinase does not readily oxidize aromatic diamines or dihydroxyphenols whose functional groups are para- related.
5. The tyrosinase enzyme system can be used in both aqueous and organic solvent systems.
6. Enzyme activity is two-fold in aqueous systems i.e. the removal of phenolic compounds from industrial wastewaters, and also the formation of polyphenolic polymerization products used widely in industry.
7. Indications of enzyme reaction-inactivation due to product binding were detected, but could not be proved experimentally.
8. A number of isoenzymes can be detected on zymogram analysis.
9. A binding-release system is thought to take place during the aerobic oxidation of phenolic compounds by tyrosinase.
10. A catecholic intermediate was detected by gas-chromatography during organic biocatalysis studies, but not during aqueous aeration experiments.
11. Zymograms cannot reliably be used as a detection system for enzyme activity in organic solvents.
12. Crude *A. bisporus* compares favourably with the commercial tyrosinase preparation. High enzyme activities were seen on assay, good removal abilities were detected and the use of the crude enzyme is more cost-effective.
13. Mushroom compost is not a source of tyrosinase producers.
14. Tyrosinase produced by mycelia in deep-liquid culture showed very good phenol removal abilities and presents a useful alternative to the large-scale production of mushroom fruiting bodies as a source of the enzyme for use in environmental phenolic wastewater treatment.

CHAPTER 6

THE USE OF WHOLE CELL SYSTEMS FOR THE TREATMENT OF PHENOLIC EFFLUENTS

Summary The use of whole cells for the removal of phenol from liquid media presents a practical alternative to complex enzyme extraction procedures. This application was investigated using *S. antibioticus*, *S. glaucescens*, *S. lividans* (pIJ702) and AECI culture no. 26. Appreciable removal rates were detected with all cultures.

6.1. INTRODUCTION

Although some success has been attained with the use of enzyme extracts in practical industrial applications, this generally requires the implementation of lengthy extraction and purification procedures. These methods are not easily applicable to industrial scale processes and extraction efficiencies and recovered enzyme activity is often low (Johnson, 1977). A simpler and more practical technique involves the *in situ* removal of phenol by whole cell preparations.

Methods involving whole cells rather than enzyme extracts have been used in a wide range of applications in industry, including the use of microbial systems for the pretreatment of effluents. *Geotrichum candidum* has been harnessed for the partial aerobic removal of phenolic inhibitor compounds in olive mill wastewaters, thereby producing an effluent that can be anaerobically biodegraded (Borja *et al.*, 1992). A South African company, AECI, recently marketed a fungal product for the removal of cyanide from aqueous wastewaters. This product, known as Cyclear, consists of dried *Fusarium* cells which degrade cyanide when added to a cyanide spill (Kenyon, pers. comm). Immobilized *Gloeocercospora sorghi* has also been used continuously in a packed-bed column reactor for several weeks for the degradation of high cyanide concentrations (Knowles and Wyatt, 1992).

Either batch or continuous culture systems can be utilized for microbial transformations. Although batch systems are more commonly used for enzyme-catalyzed transformations, continuous systems are popular for whole cell fermentations. However, the optimization

of a continuous culture system requires the maintenance of a fine balance between biomass production and the enzymatic transformation of the substrate. Batch systems often serve as a preliminary indication of a selected transformation system's feasibility.

The primary advantage in utilizing a whole cell system lies in the ease of separating the biocatalyst and product after catalysis. These systems may rely on the primary metabolism of the cell for necessary cofactors and make use of the transport systems of the cell. The permeability of the cells to substrate and product may form a major barrier since it is improbable that both are subject to active transport. Transport may therefore play a rate-limiting role in the biotransformation process (Rosevear and Lambe, 1983).

Enzyme instability and the provision of reducing power to the system may be overcome by employing whole organisms (Best and Higgins, 1983). This was of particular interest to this study due to the apparent instability of *Streptomyces* tyrosinase. A major contributing factor to enzyme instability is inactivation during the oxidation of various phenolic compounds. This does not take place in living fungal cultures (Fahraeus and Ljunggren, 1961).

Using whole cell systems mimics conditions in natural environments, thereby providing cellular enzymes which may be required for the pretreatment of a substrate. It is assumed that although only one suite of enzymes may degrade a particular substrate, other enzymes may be utilized to prepare the substrate for biodegradation.

The advantages and disadvantages of using enzyme extracts or whole cells, as listed by Davies *et al.* (1989) and determined by this research programme, are summarized below:

Enzyme extracts

Simpler apparatus is needed when using enzyme extracts for biotransformations, and the process is easier to control and monitor. Enzymes are very specific for selected reactions, but disadvantages include the expense of extraction and the addition of enzyme cofactors if necessary.

Whole cells

Disadvantages include the possible expense of modified fermentation equipment, side reactions which may interfere with the biotransformation process, and difficulties encountered in removing spent cells and products from the medium. Factors important to this study are problems associated with transporting substrates, products and oxygen into and out of the cells.

Advantages include the cost effectiveness of production since for example enzyme cofactors which may be required by the enzymes are present in the cells. Expensive enzyme extractive procedures are also not required. An important advantage to using whole cells for phenol removal is the protection of the polyphenolase system within the cell, therefore reducing enzyme inactivation and promoting enzyme stability. Enzymes are also protected from denaturants which may be present in the effluent.

Two different whole cell systems can be employed for wastewater treatment. Cells may be used as enzyme "packages" which are added to effluents for the immediate removal of pollutants, or as actively growing cultures consistently removing contaminants over time. The objective of this research was to investigate these two systems for the treatment of phenolic effluents.

Successful polyphenolase producers were identified in the foregoing survey and phenol removal efficiencies evaluated using enzyme extracts. This study was initiated to examine phenol removal abilities by whole cell systems, both with and without the addition of growth medium for the maintenance of cell biomass to compare the "enzyme package" versus the "actively growing culture" effects.

6.2. MATERIALS AND METHODS

As methods reported in this study are different from methods previously used, they have been described separately. Methods in Chapter 2 are referred to when used.

6.2.1. The selection of microorganisms

Cultures were selected for this study based on previous determinations of tyrosinase activity against L-tyrosine (AECI culture no. 26) or L-DOPA (Streptomyces). Optimum enzyme activities are expressed for both substrates using the conversion factor as determined by *A. bisporus* tyrosinase assayed against L-tyrosine and L-DOPA (Table 6.1).

Table 6.1: Cultures selected for whole cell fermentation studies and their respective activities against L-tyrosine and L-DOPA, expressed as units of enzyme activity.ml⁻¹.

CULTURE	ASSAY SUBSTRATE	
	L-tyrosine	L-DOPA
AECI culture no. 26	1 607.7	303.2
<i>S. antibioticus</i>	2 653.2	500.6
<i>S. glaucescens</i>	2 365.9	446.4
<i>S. lividans</i> (pIJ702)	148.4	28.0

6.2.2. Enzyme production in liquid media

Microorganisms were cultured in selected shaker-flask media (Table 6.2) so as to induce optimum enzyme production. These methods are more clearly outlined in section 2.3.1 and appendix 3 for AECI culture no. 26, section 2.4.2.2 (experiment b) and appendix 4 (GYM medium) for *S. antibioticus* and *S. glaucescens*, and section 2.4.2.4 and appendix 4 (MMT medium) for *S. lividans* (pIJ702). Table 6.2 summarizes enzyme-producing conditions for each culture.

Table 6.2: Culture media and conditions of growth for optimum enzyme production by cultures selected for whole cell fermentations.

CULTURE	GROWTH MEDIUM	CONDITIONS OF GROWTH
AECI culture no. 26	0.05% phenolics in glucose-phenol media	Incubated shaking at 37°C for 7 days
<i>S. antibioticus</i>	pH 7.2 GYM	Incubated shaking at 30°C for 20 hours, 25°C for 24 hours
<i>S. glaucescens</i>	pH 7.2 GYM	Incubated shaking at 30°C for 20 hours, 25°C for 24 hours
<i>S. lividans</i> (pIJ702)	pH 7.6 MMT	Incubated shaking at 30°C for 5 days

Once the optimum enzyme-producing phase of growth had been reached, cell pellets and culture medium were separated by centrifugation at 4°C for 20 minutes at 10 000 rpm. Microbial pellets were subsequently used for the inoculation of phenolic broth media. Since extracellular enzymes are produced within the cell and secreted into culture medium, pellets of the predominantly enzyme-secreting wild-type *Streptomyces* were also utilized.

6.2.3. The preparation and inoculation of phenolic media

Two media systems were evaluated for each culture, a simulated phenolic effluent i.e. phenolic buffer, and phenol-containing growth medium. 200ml of each medium was dispensed into 500ml Erlenmeyer flasks and the appropriate quantity of phenol added. Phenol concentrations used were 0.01%, 0.1% and 1.0%. Uninoculated controls were run for each system. Phenolic buffer media was prepared in pH 8 phosphate buffer, inoculated with microbial pellets and incubated shaking at 37°C for AECI culture no. 26, and 30°C for the *Streptomyces*.

The preparation of phenol-containing growth media was based on results acquired in chapters 3 and 4:

1. AECI culture no. 26: Glucose-phenol minimal media was prepared (appendix 3) to the required phenol concentration, media buffered to pH 7.2, inoculated and incubated shaking at 37°C for 1 week.
2. *S. antibioticus* and *S. glaucescens*: GYM media was prepared (appendix 4), phenol added, media buffered to pH 7.2, inoculated and incubated shaking at 30°C for 20 hours. Additional nutrients were added after 20 hours as in section 2.4.2.2, and flasks thereafter incubated at 25°C for 1 week.
3. *S. lividans* (pIJ702): MMT media was prepared (appendix 4), phenol added, media buffered to pH 7.6, inoculated and incubated shaking at 30°C for 1 week.

Experiments were carried out in duplicate and results expressed as a mean value.

All flasks were sampled after inoculation ($t=0$), 6 hours later ($t=6$ hrs), and then once every 24 hours for one week. 5ml samples were taken and biomass determinations carried out by dry weight analysis as described in section 2.4.2.2. The concentration of phenol in each sample was determined by analysis on GC using a Tenax packed column (section 2.20.2.1). Phenol removal efficiencies were verified by analysis on a Waters Dimension 1 GC at Protea Pharmaceuticals (East London, RSA). A J+W DB-wax (15m, 1 μ m film) column was used under the following conditions:

Injection temperature	200°C
FID temperature	250°C
Nitrogen (carrier gas) flow	30.0 ml.min ⁻¹

The temperature programme used was as follows:

120°C for 1 minute, followed by an increase to 170°C at 20°C.min⁻¹ and held for 0.5 minutes, and another increase to 200°C at the same rate and held for another minute.

6.3. RESULTS

Figures 6.1 - 6.8 compare growth in phenol-containing growth medium and phenol in buffer without nutritional growth requirements added.

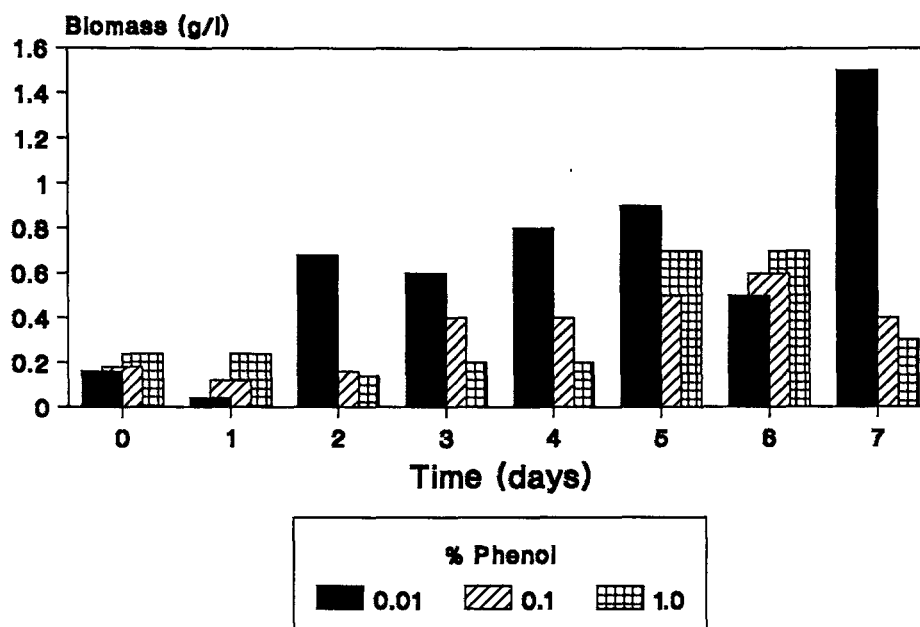


Figure 6.1: Biomass production of AECI culture no. 26 in phenol-containing growth medium.

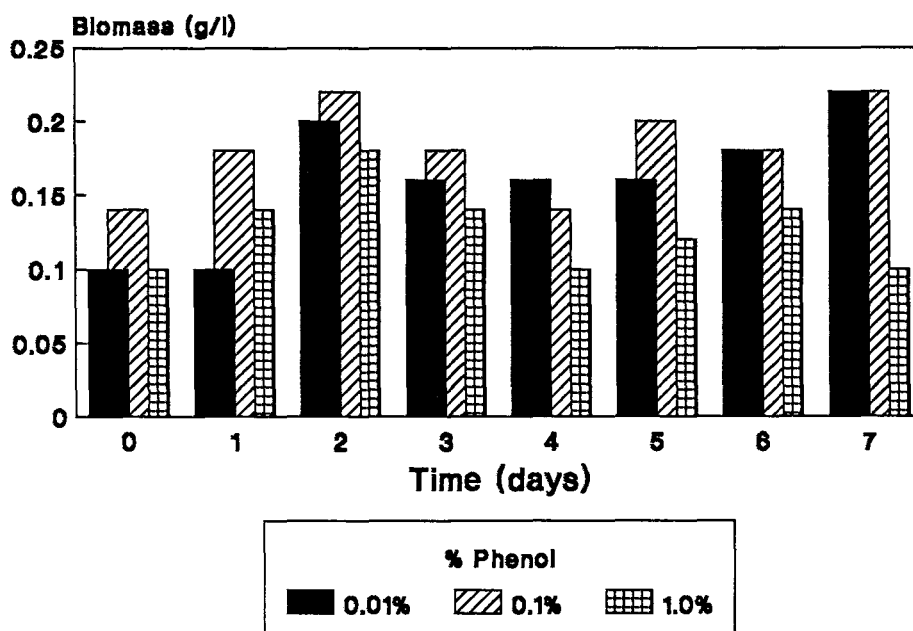


Figure 6.2: Biomass production of AECI culture no. 26 in phenolic buffer.

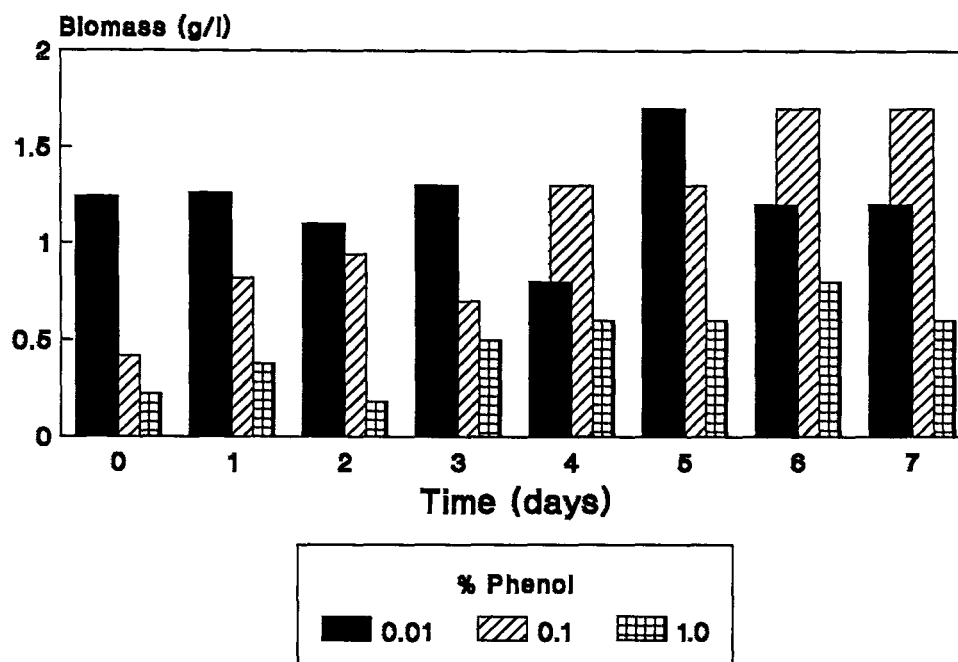


Figure 6.3: Biomass production of *S. lividans* (pIJ702) in phenol-containing growth medium.

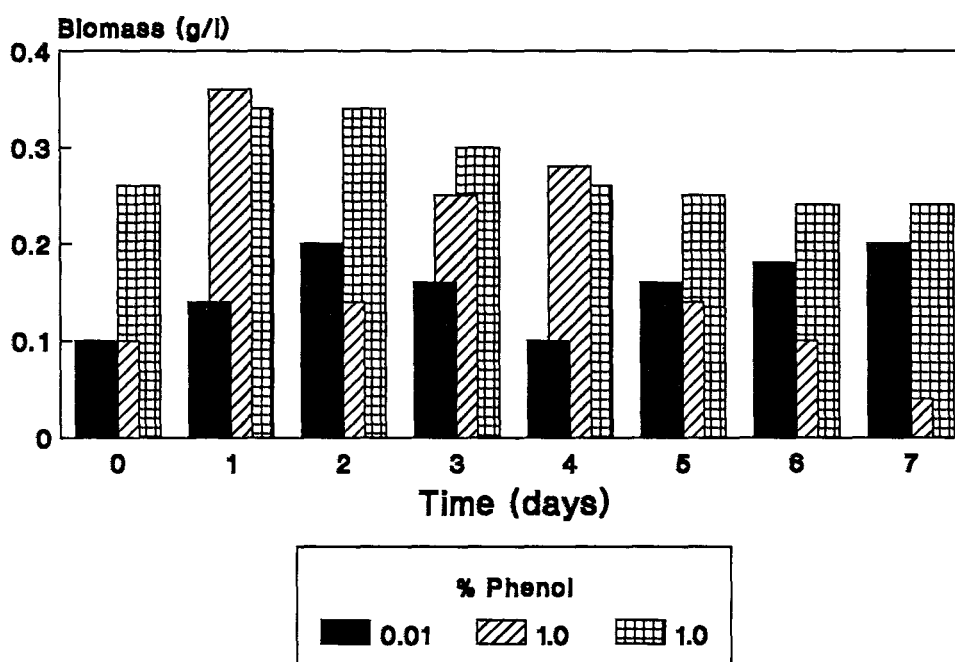


Figure 6.4: Biomass production of *S. lividans* (pIJ702) in phenolic buffer.

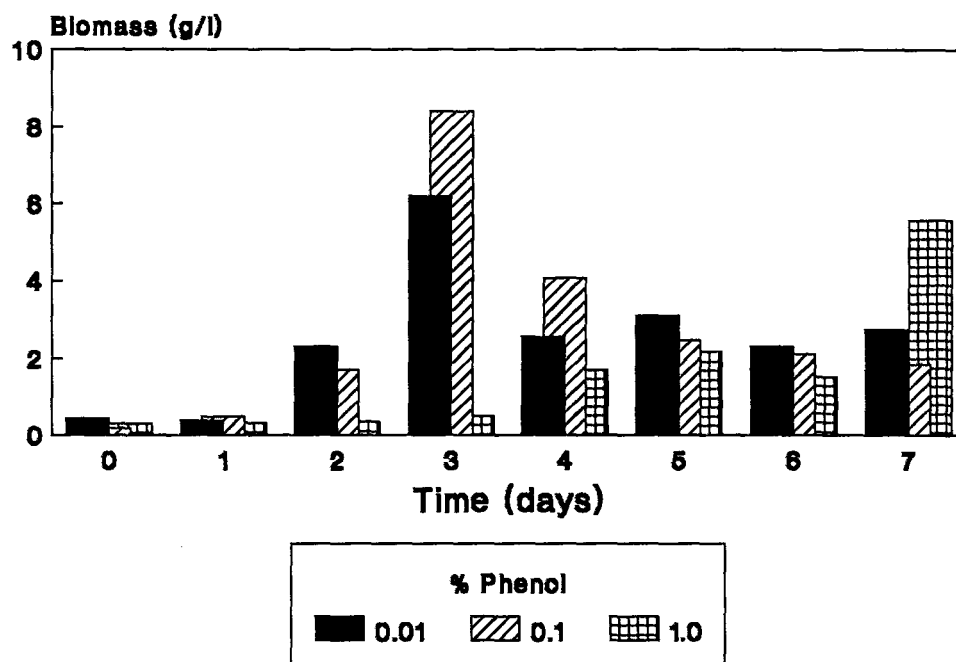


Figure 6.5: Biomass production of *S. glaucescens* in phenol-containing growth medium.

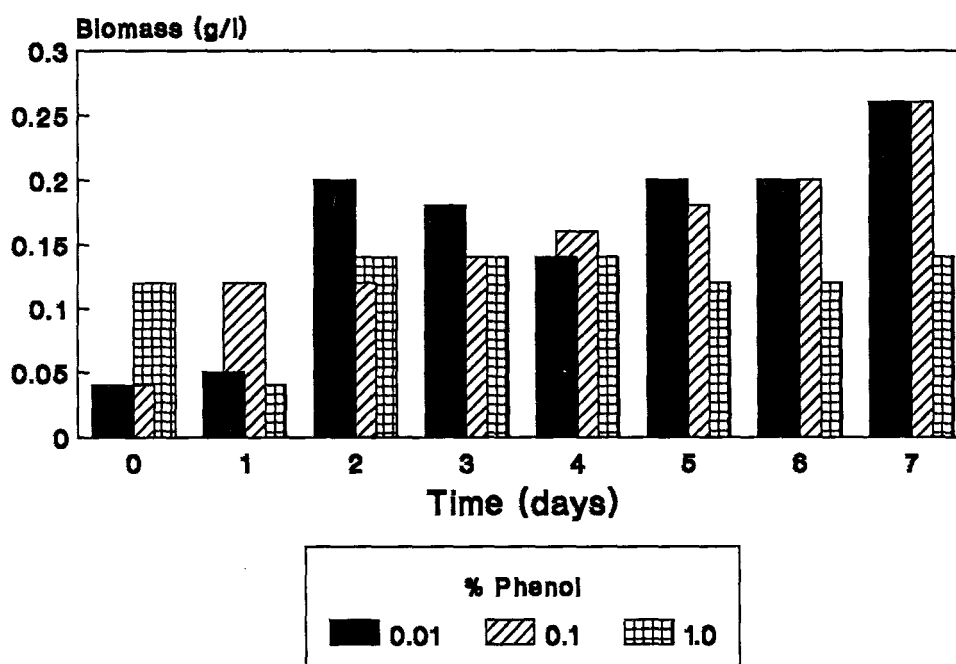


Figure 6.6: Biomass production of *S. glaucescens* in phenolic buffer.

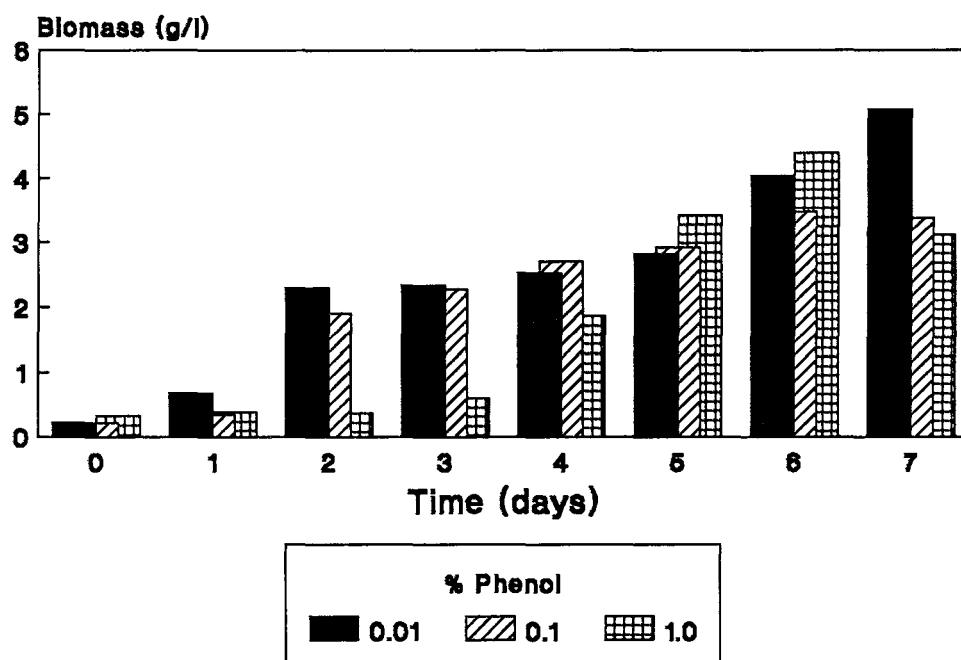


Figure 6.7: Biomass production of *S. antibioticus* in phenol-containing growth medium.

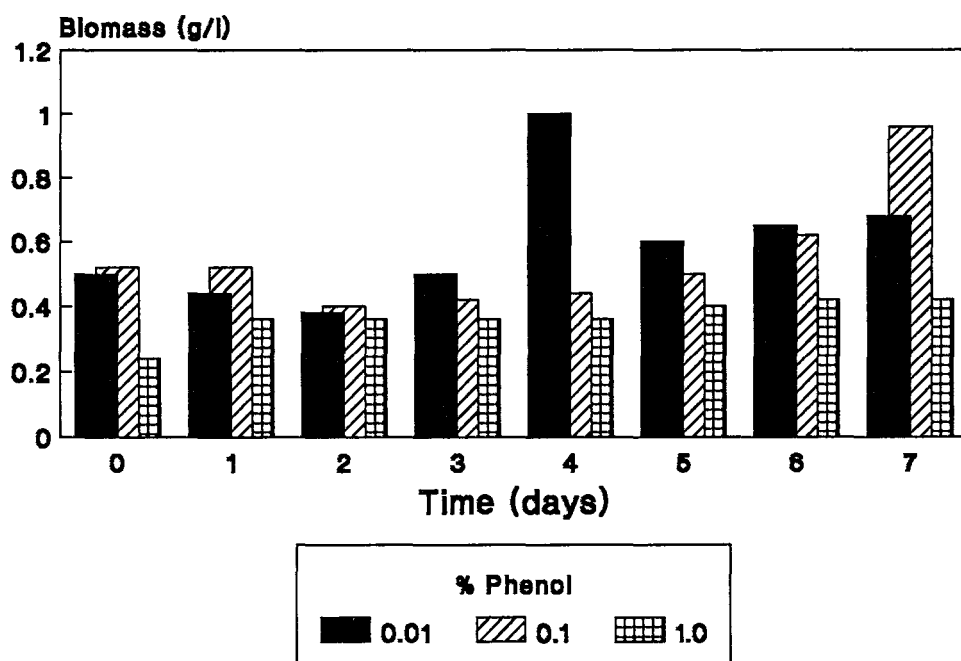


Figure 6.8: Biomass production of *S. antibioticus* in phenolic buffer.

Growth was seen at all concentrations of phenol, with 1% phenol generally showing the least growth. Although less growth was seen in phenolic buffer flasks than in growth media, biomass production was significant. This may be due to phenol consumption as sole carbon source. The increase in biomass production per system is presented in table 6.3.

Table 6.3: Increase in biomass production after 7 days, expressed as a percentage.

[Phenol] in medium	AECI no. 26		<i>S. lividans</i> (pIJ702)		<i>S. glaucescens</i>		<i>S. antibioticus</i>	
	g-p	buffer	MMT	buffer	GYM	buffer	GYM	buffer
0.01	838	120	0	43	552	550	2 209	36
0.1	122	11	210	0	922	500	1 436	85
1.0	25	0	173	0	1 760	0	875	75

g-p: glucose-phenol growth medium

With the exception of *S. glaucescens*, biomass production in phenolic buffer was approximately 10% that of growth in phenol-containing culture media, demonstrating the effect of additional carbon sources on biomass production. Although phenol was shown to have a deleterious effect on biomass production by the wild-type Streptomyces - *S. antibioticus* biomass increases 6.7x more after 44 hours in phenol-free pH 7.2 GYM medium than after 7 days in 0.01% phenol, and *S. glaucescens* 10x more after 35 hours in phenol-free medium (Chapter 4) than after 7 days growth in 1.0% phenol - growth in phenol-containing culture media was good. *S. lividans* (pIJ702) cultures are less affected by phenol; growth in a phenol-free medium after 7 days was only 1.7x that in phenol-containing growth medium.

Phenol removal is graphically represented in figures 6.9 - 6.13. Removal efficiencies recorded were good, confirming the selection of these organisms as phenol-tolerant phenol-removers. Phenol removal efficiencies for the whole cell systems were determined by GC analysis and are tabulated in table 6.4.

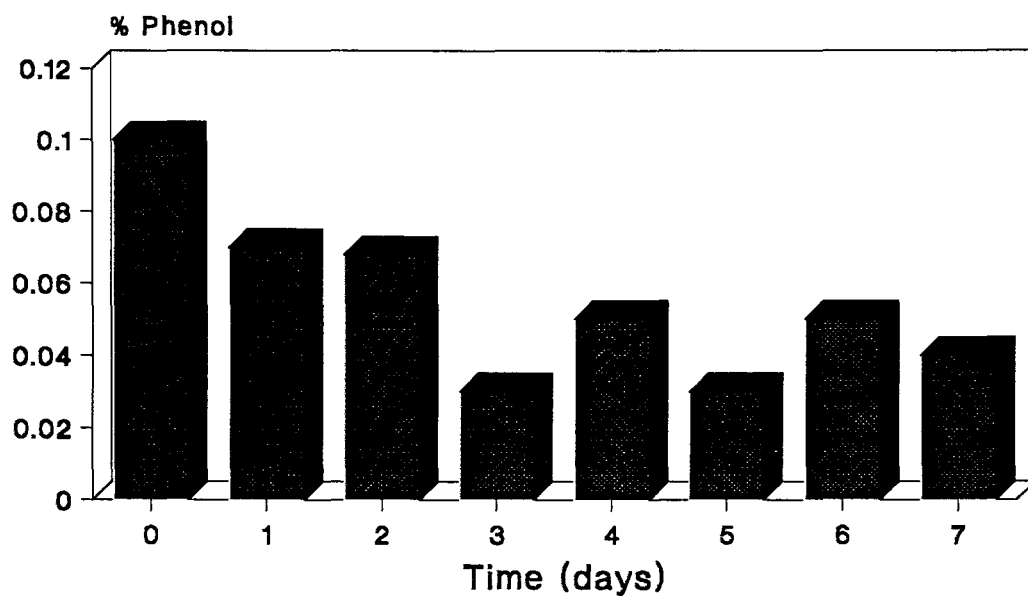


Figure 6.9a: Changes in phenol concentration of AECI culture no. 26 0.1% phenol-containing growth medium as monitored by GC.

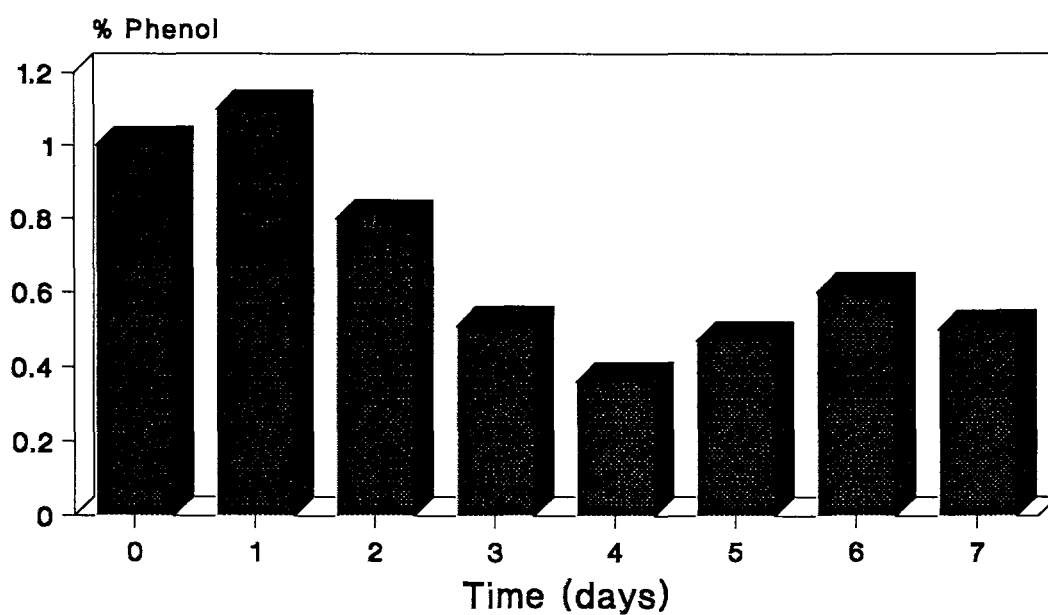


Figure 6.9b: Changes in phenol concentration of AECI culture no. 26 1% phenol-containing growth medium as monitored by GC.

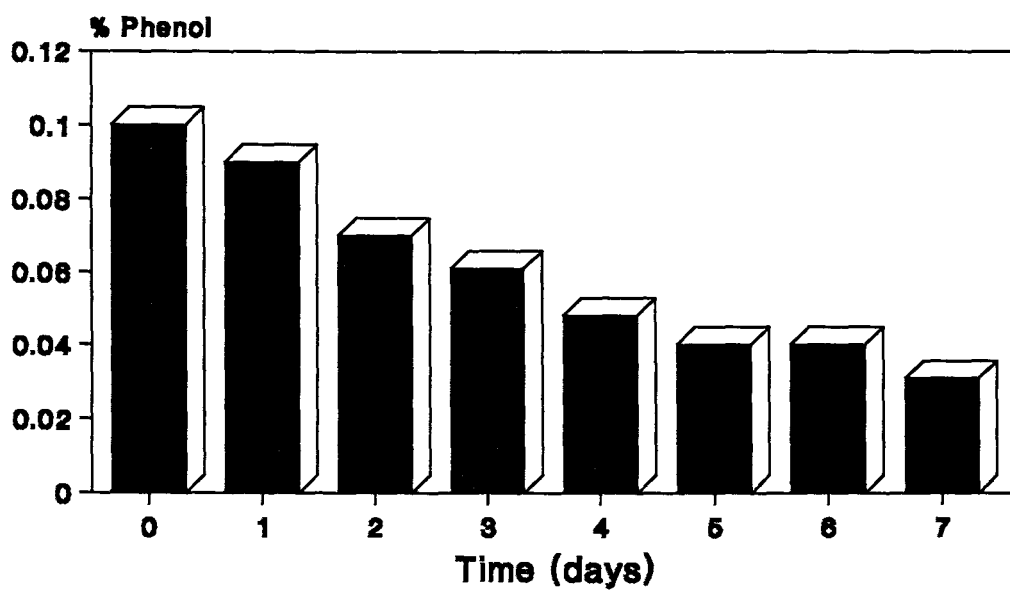


Figure 6.10a: Changes in phenol concentration of *S. lividans* (pIJ602) 0.1% phenol-containing growth medium as monitored by GC.

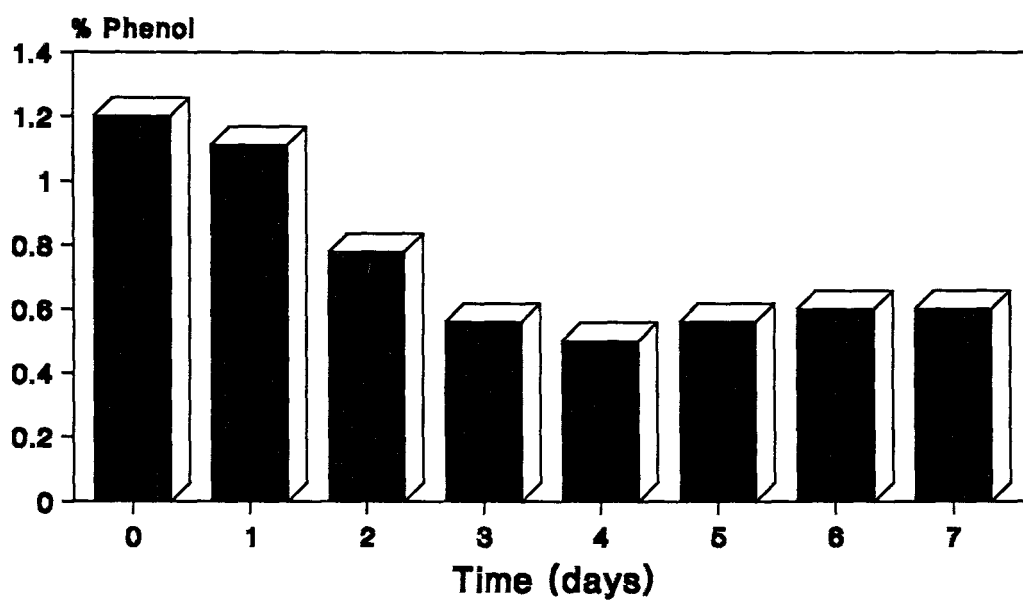


Figure 6.10b: Changes in phenol concentration of *S. lividans* (pIJ702) 1% phenol-containing growth medium as monitored by GC.

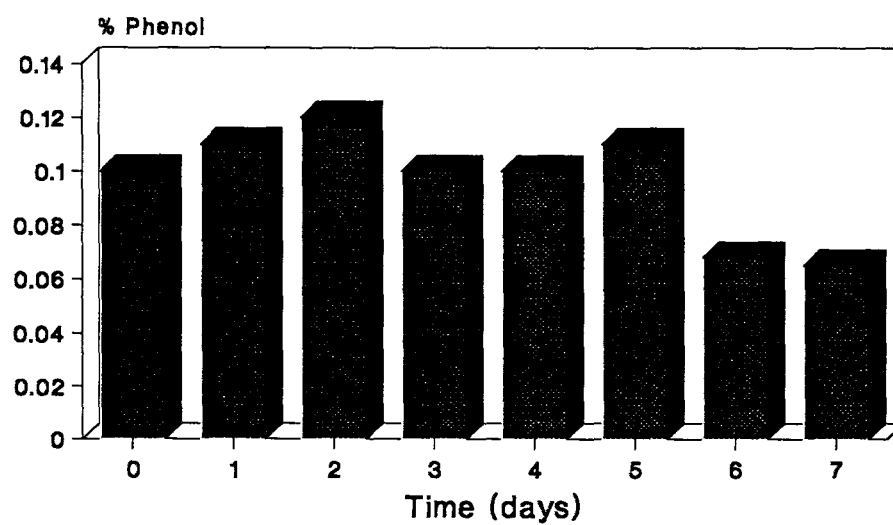


Figure 6.11a: Changes in phenol concentration of *S. glaucescens* 0.1% phenol-containing growth medium as monitored by GC.

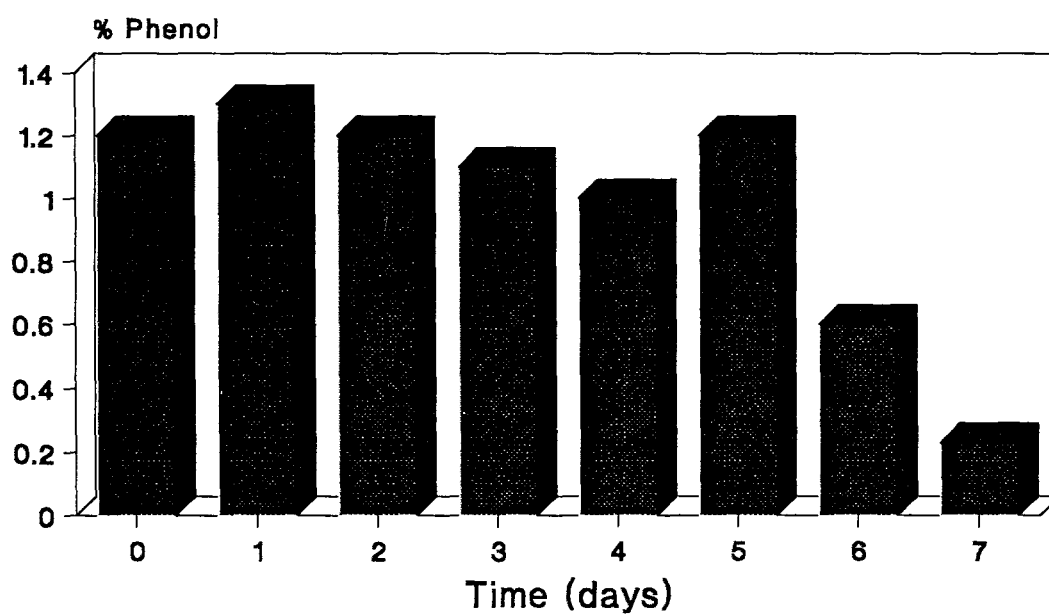


Figure 6.11b: Changes in phenol concentration of *S. glaucescens* 1% phenol-containing growth medium as monitored by GC.

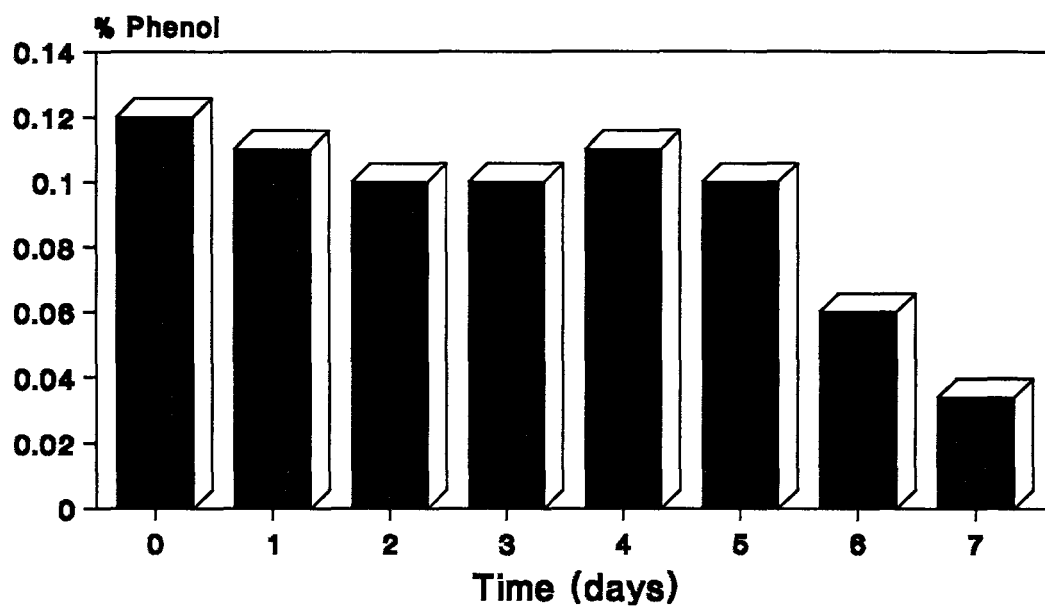


Figure 6.12a: Changes in phenol concentration of *S. antibioticus* 0.1% phenol-containing growth medium as monitored by GC.

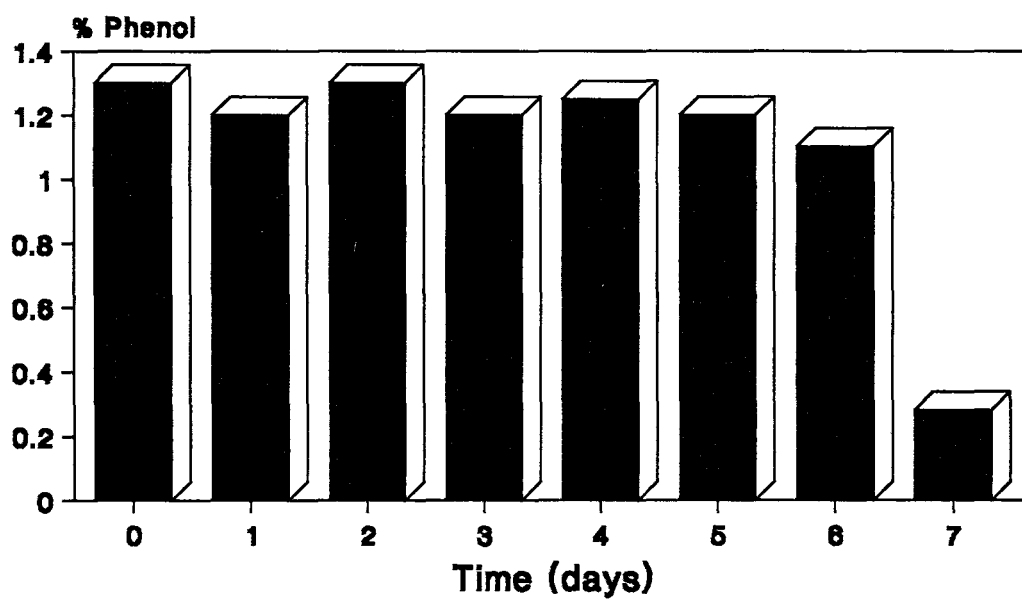


Figure 6.12b: Changes in phenol concentration of *S. antibioticus* 1% phenol-containing growth medium as monitored by GC.

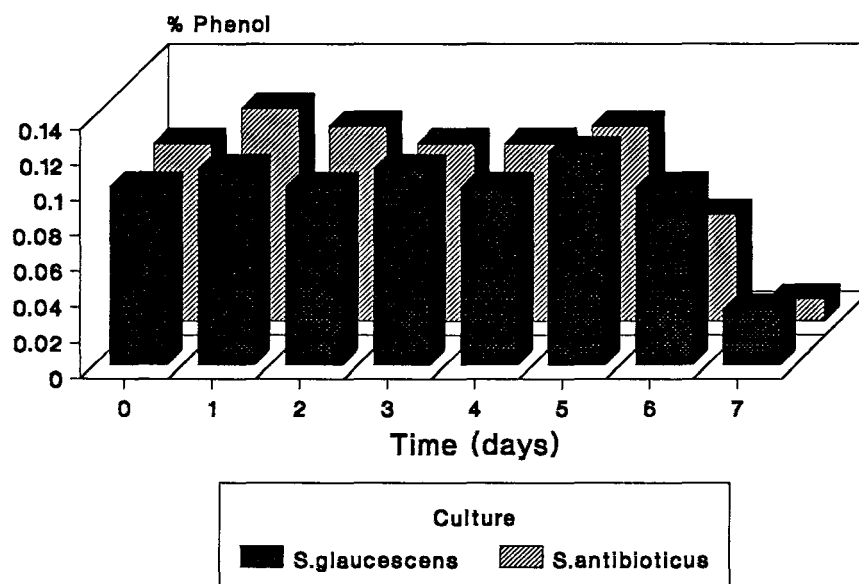


Figure 6.13a: Changes in phenol concentration of *S. antibioticus* and *S. glaucescens* 0.1% phenolic buffer.

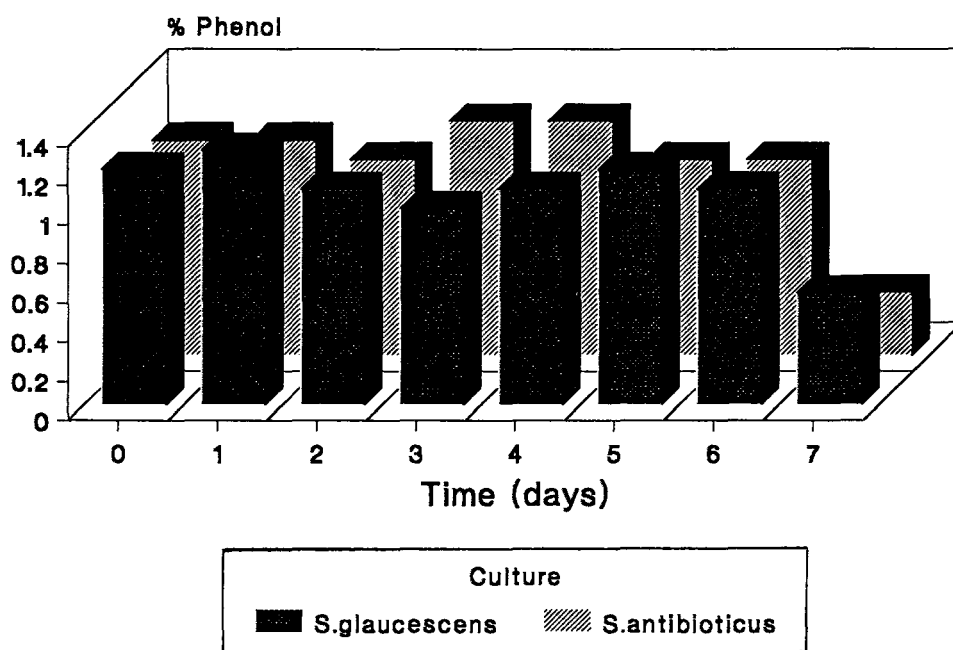


Figure 6.13b: Changes in phenol concentration of *S. antibioticus* and *S. glaucescens* 1% phenolic buffer.

Table 6.4: Phenol removal efficiencies (expressed as % phenol removed) after 7 days as determined by GC analysis.

[Phenol] in medium	AECI no. 26		<i>S. lividans</i> (pIJ702)		<i>S. glaucescens</i>		<i>S. antibioticus</i>	
	g-p	buffer	MMT	buffer	GYM	buffer	GYM	buffer
0.01	0	0	0	0	0	0	0	0
0.1	60	0	60	0	32	69	66	88
1.0	46	0	40	0	77	43	72	68

g-p: glucose-phenol growth medium

6.4. DISCUSSION

AECI culture no. 26 and *S. lividans* (pIJ702) showed little growth in phenol buffer, and subsequently little enzyme production was seen, suggesting that phenol was not mobilized as a carbon source. There does appear to be an association between the growth of *S. antibioticus* and *S. glaucescens* buffer cultures and tolerance to various concentrations of phenol. This correlation was previously suggested (Chapter 4), but requires verification by ¹⁴C-labelling experiments.

Although good growth was seen in 0.01% phenol cultures, most cultures did not show any phenol removal. Reduced growth rates in 0.1% and 1% phenol is probably due to the increasing toxicity of phenol to the microbial cell. However, biomass production at these concentrations disproves Dagley's (1985) claim that growth generally does not take place at levels above 0.05% phenol. This study has therefore identified a number of phenol-tolerant microbial cultures.

AECI no. 26 and *S. lividans* (pIJ702) cultures both displayed early phenol removal occurring in the first few days. The wild-type Streptomyces showed sudden phenol removal occurring only after 6 or 7 days. This applied to both growing and non-growing cultures. Removal should therefore be investigated in a continuous system given that cultures require a period of adaptation before enzyme production begins and phenol removal is initiated.

With the exception of *S. glaucescens*, all cultures showed either reduced removal on increasing phenol concentrations in growth media (AECI culture no. 26 and *S. lividans* (pIJ702)), or similar removal ability in 0.1% and 1% phenol (*S. antibioticus*). The reaction of AECI culture no. 26 and *S. lividans* (pIJ702) indicates an inhibitory mechanism present at the higher phenol concentration, verified by decreasing biomass production (Table 6.3). Although the addition of phenol to growth medium appears to reduce growth rates of wild-type Streptomyces, an increase in phenol concentration from 0.1% to 1% did not inhibit phenol removal. The removal ability of *S. antibioticus* was not significantly affected, while *S. glaucescens* responded favourably by increasing its growth and phenol removal ability. Results appear to indicate increased tolerance to phenol in the medium.

S. antibioticus and *S. glaucescens* showed a decrease in phenol removal ability with increasing phenol concentrations in phenolic buffer, and an increase in removal in phenolic growth media. This seems to indicate that in the presence of additional carbon sources (contained in the growth medium), removal of higher phenol concentrations takes place and phenol may be mobilized as a carbon source. The provision of other essential nutrients, such as methionine contained within the yeast extract of GYM medium, should not be overlooked. The addition of growth media therefore has a positive effect on phenol removal and enables removal at higher phenol concentrations.

The use of both enzyme extracts and whole cells for the successful removal of phenol from liquid media has been demonstrated. The removal of phenolic compounds from aqueous wastewater systems would present a valuable industrial application of these whole cell fermentation systems. The Streptomyces soil bacteria would also be ideally suited for the removal of environmental phenolic pollutants e.g. during phenol spills. Although removal was only tested against phenol, more complex phenolic compounds could increase the rate of biocatalysis due to the positive effect of substrate side-chains on enzyme reaction rates (reported by Harrison *et al.*, 1967 for mushroom tyrosinase). However, Philipp *et al.* (1991) reported *S. michiganensis* DSM 40015 tyrosinase to be very restrictive in regard to the aromatic part of its substrate, i.e. only one additional substituent prevented its oxidation. They concluded that *Streptomyces* tyrosinase cannot be used for the detoxification of aromatic compounds. Although the present study only investigated removal against a monophenolic substrate, all evidence suggests that the Streptomyces are successful phenol removers. *S. michiganensis* enzyme extracts (Philipp *et al.*, 1991)

showed very low activity against phenol, but whole cell systems were not examined. The results obtained by this study strongly support the use of Streptomyces whole cells, not enzyme extracts, for the detoxification of phenolic effluents.

6.5. CONCLUSIONS

The use of whole cells for phenol removal appears to be superior to that of extracted tyrosinase, due to the improved stability of the enzyme system. The protection offered by the cell reduces enzyme inactivation by processes such as substrate inhibition and product binding.

S. lividans (pIJ702) enzyme extracts could not be utilized for phenol removal studies due to extensive melanin binding, while *S. antibioticus* and *S. glaucescens* extracts were too unstable. In addition, the time period of aeration experiments (i.e. 6 hours) was too short to show any phenol removal by these organisms due to the slow oxidation of phenol by *Streptomyces* tyrosinase. AECI culture no. 26 enzyme extracts showed a higher phenol removal efficiency than whole cell systems. Phenol removal ability per mg of cells per system was as follows:

Enzyme extract in 0.05% phenolic buffer - 2.4% phenol removed.

Whole cells in 0.1% phenolic growth medium - 0.75% phenol removed.

Whole cells in 1% phenolic growth medium - 0.67% phenol removed.

Unfortunately, the effect of increasing phenol concentrations on extracts were not ascertained.

In conclusion, good removal efficiencies were recorded using whole cell fermentation systems, indicating their potential application in industry for the effective treatment and removal of phenolic contaminants from wastewaters. The potential also exists for these systems to be used in the removal of environmental phenolic pollutants e.g. phenol spills.

CHAPTER 7

GENERAL DISCUSSION

The use of microbial enzymes for biotransformations and the biodegradation of environmental pollutants is of relevance to both the theoretical and applied sciences. Microbial enzymes are generally more useful than animal and plant enzymes due to the wide variety of catalytic qualities that can be optimized by fermentation techniques. Cheap and abundant enzyme supplies can often be produced in liquid culture, and microbial enzymes are generally more stable and safer due to a low allergenic potential. Optimization studies are also more easily carried out on microbial cells owing to their shorter generation times and relative ease of screening (Wiseman, 1986).

A range of selected microorganisms were evaluated in this study for the production of polyphenolase enzymes. This enzyme system can potentially be applied in both aqueous and organic media for the purification of phenolic effluents and the production of fine chemicals, respectively. The evaluation and development of rapid and effective bioassay methods for determining the presence of polyphenolase enzymes in large numbers of environmental isolates formed an important part of the study. Once potential polyphenolase producers were identified, an attempt was made to optimize complex growth requirements that would favour enzyme production. These systems were then assayed for phenol-removing potential in a simulated phenol-containing effluent.

Microorganisms showing good phenol removal ability generally originate from environments where tyrosinase may be useful for the utilization of complex substrates, e.g. Streptomyces which occur in soil and aquatic environments and bacteria and fungi associated with lignocellulose biodegradation. In this study promising isolates included cultures isolated from rotting timber i.e. Hogsback culture no. 1, and organisms isolated from the vicinity of a coal gasification plant i.e. AECl culture no. 26.

Growth optimization and enzyme production

The manipulation of an organism's environment for the production of a single commodity is aided by its ability to easily respond to changing environmental conditions. In order to flourish in natural ecosystems, microorganisms have to cope with wide fluctuations in pH, temperature and nutrient availability. Since the expression of the genotype into the phenotype is largely mediated by enzymes, it is not surprising that changes in the environment can result in wide variations in enzyme activity. It is well documented that certain microbial characteristics can change markedly with changes in growth conditions (Melling, 1977). It is therefore essential for organisms to regulate enzyme synthesis so as to control enzyme production to suit the prevailing conditions. This control is achieved by regulatory mechanisms such as induction and catabolite repression. Discrepancies between growth and enzyme induction of different organisms on various substrates may provide a demonstration of the efficiency of catabolite repression. When a microorganism is harnessed for the production of a commodity, these regulatory mechanisms must be understood in order to shift metabolic processes towards its production.

As enzyme production is generally biomass-dependent, emphasis must be placed on the composition of the growth media and the level of growth-limiting nutrients. Besides macronutrients (e.g. carbon, nitrogen, potassium and phosphorous at concentrations greater than 10^{-4} mol.l⁻¹) and micronutrients (required at concentrations less than 10^{-4} mol.l⁻¹), other specific trace-elements may be required under specialized conditions, e.g. copper which is essential for the production of catalytically active tyrosinase (Dunn, 1985).

In order to accommodate fluctuations in nutrient availability in the natural environment, organisms can regulate the synthesis of certain enzymes used to assimilate a potential substrate. In this way, the exclusion of carbon sources other than phenol (e.g. glucose) from growth media will favour the induction of the ortho- or meta- suite of degrading enzymes, rather than the tyrosinase enzyme system (Spänning and Neujahr, 1990). The phenolic substrate is then assimilated into cell components, rather than dissimilated (i.e. changed into less toxic end-products). If glucose is included in the culture medium, ortho- or meta- enzymes will catalyze its assimilation, leaving tyrosinase to degrade phenol. The type of enzyme induced to degrade a substrate therefore depends on the nature of the

substrate and the energy required to carry out the process. In batch cultures there is always the problem of initially adding too little inducer, which is then degraded immediately and the culture grows in the absence of the inducer, or too much inducer which can cause sterility or death of the culture. The role of phenol as a limiting nutrient and inducer for the detoxification of phenolic waste has been researched with limited success (e.g. Jones and Carrington, 1972). Little information is available on variations in enzyme activities associated with this substrate (Melling, 1977).

The importance of oxygen for tyrosinase production was illustrated in Streptomycete growth optimization studies. Aeration must be carefully maintained as the oxygen level drops once enzyme production starts and is at a low concentration throughout the enzyme production phase (Aunstrup, 1977). The presence of filamentous fungi and Streptomycete mycelia in submerged cultures also has a profound effect on oxygen transfer and aeration efficiency. In bacterial fermentations, total oxygen uptake reaches a maximum value at the time of oxygen limitation and remains so until another nutrient becomes limiting. In the case of mould fermentation, the total oxygen uptake rate decreases during the period of oxygen limitation due to the increasing viscosity of the culture, caused by increasing mycelial concentrations. The branched mycelia form a dense, interwoven network which display viscous non-Newtonian properties. The age of the culture also influences its properties. Banks (1977) reported that a culture of *Streptomyces griseus* displayed Newtonian behaviour in the initial stages of fermentation owing to a low biomass concentration and also in the final stages due to mycelial fragmentation and lysis, while non-Newtonian behaviour was observed mid-way through the fermentation.

Further oxygen deprivation in fungal and Streptomycete cultures is brought about by the formation of pellets in liquid media. When an organism forms filamentous mycelia in submerged culture, all the hyphae are exposed to the medium and growth is exponential. When pellets are formed, nutrients cannot diffuse into the pellet fast enough to maintain the growth of the whole culture. Upon reaching a particular diameter, the outside shell of the pellet may continue growing exponentially, but the remaining biomass stops growing and anaerobiosis may develop inside the pellet (Oliver and Trinci, 1985). Although the present study showed a correlation between enzyme production and biomass formation, the transfer of oxygen and inducers to the inside of the cell may have been inadequate for the

induction of high enzyme concentrations. The effect of mycelial pellet formation on nutrient and oxygen diffusion could therefore explain the low biomass production and glucose utilization shown by Streptomyces cultures in liquid media. The maintenance of filamentous mycelia in *A. bisporus* deep-liquid cultures aided enzyme production by ensuring a flow of nutrients, inducers and oxygen into the cell. Continuous passaging prevented pellet formation and therefore promoted enzyme production. Pellets are, however, easier to recover and require less power input to the fermentation (Aunstrup, 1977).

Although phenols and catechols are fairly common microbial metabolites, they nevertheless become toxic even to the microorganisms that degrade them. Growth therefore seldom takes place on phenol concentrations above 0.5 g.l⁻¹ (Dagley, 1985). Almost 40% of cultures screened during this study grew on 0.1% phenol-containing agar (Chapter 3). AECI culture no. 26 and the Streptomyces showed good growth in liquid media containing up to 1% phenol (Chapter 6). Two reasons have been offered for microbial growth on aromatic compounds: firstly, to enzymatically prepare the substrate for utilization as a nutrient, and secondly, unspecific enzymes recognize chemical structures resembling those of natural substrates (Dagley, 1985). Microbial communities generally evolve to utilize certain contaminants as carbon and energy sources when under long-term selection pressure from chronic contamination (Ridgway *et al.*, 1990). A third proposal has resulted from the work of Cerniglia and Gibson (1979, 1980) (cited in Dagley, 1985) on the filamentous fungus *Cunninghamella elegans*, which oxidizes the environmental carcinogen, benzo[a]pyrene. It appears that the fungus recognizes the chemical as a xenobiotic requiring detoxification.

High enzyme production was noted in *A. bisporus* mycelial cultures, AECI culture no. 26 and the Streptomyces. Growth optimization is more easily accomplished with bacteria than fungi, as bacterial growth rates and protein contents are higher and culture media can be efficiently aerated. Undesirable metabolites (e.g. mycotoxins) are seldom formed and bacteria are generally more genetically stable than fungi (Solomons, 1985). Advantages of fungal cultures include low nutritional requirements and the ability to degrade complex substrates. However, both fungi and bacteria vary widely in their growth and compositional characteristics.

Standardizing growth and enzyme-producing conditions is hindered by this variation between cultures and also within species of the same culture. For example, amino acids are required for enzyme production in the Streptomyces, but depress the formation of *Azotobacter* polyphenol oxidase (Shivprasad and Page, 1989). The success of a screening programme depends on both the organisms used and the methods for detection of activity. Strain selection has a 30-40% influence on the results and the test procedure 60-70% (Crueger and Crueger, 1984). The initiation of an effective screening programme for tyrosinase producers is therefore hindered by the species specificity of tyrosinase induction. Another complicating factor is the possibility of catabolite repression of enzyme induction by constituents of the growth medium, e.g. the repression of *A. bisporus* laccase by ammonium in the medium (Kalisz *et al.*, 1986). Substrates which have been reported to bring about catabolite repression include both carbon and nitrogen sources (Crueger and Crueger, 1984). The cell normally produces enzymes to catabolize the best carbon substrate present (usually glucose), while the synthesis of enzymes utilizing other substrates is repressed until the primary substrate is exhausted (Britz and Demain, 1985). Even in the case of glucose exhaustion, ortho- and meta- enzymes (if present) would probably be induced to assimilate phenol into cellular constituents.

Enzyme induction studies

The system harnessed for enzyme induction depends on the microorganism screened. The use of methionine induction for *Streptomyces* tyrosinase was based on published reports (Crameri *et al.*, 1982; Katz and Betancourt, 1988); existing methods were adapted for the optimization of enzyme production. A number of inducing conditions were tested for other organisms within the screening programme, e.g. the inclusion of phenol or metabolic inhibitors into culture media, and the sparging of growth media with ethylene. As some organisms represented new isolates, literature was not available on their potential tyrosinase content and the optimization of its production. Inducing conditions were thus selected and applied with varying success (Scherman, 1990). Ethylene did not show any inducing ability, while oxytetracycline showed improved tyrosinase production by Hogsback culture no. 1 on assay. However, this extract demonstrated little phenol removal ability in liquid media. The correlation of growth on phenolic agar plates, assay results and the efficiency of phenol removal during aeration, proved difficult. Growth on phenol agar indicated the

ability of the microorganism to survive on a phenolic substrate, but the enzyme systems employed to achieve this growth were not identified. Enzyme assays were then used to identify tyrosinase; assays against tyrosine were more specific than those against phenol. As enzyme assays were based on enzyme-substrate activity, it was not possible to differentiate between enzyme production and enzyme activity. Copper is required for the formation of catalytically-active tyrosinase (Harrison *et al.*, 1967). Copper was in fact included in the growth media, it was thus assumed that the enzyme activity observed is representative of total enzyme production.

The detection of tyrosinase activity

Three techniques were examined for their ability to detect enzyme activity, i.e. enzyme assays, spot tests and zymogram analyses. A range of monophenol and dihydroxyphenolic assay substrates were tested. Although inappropriate for crude enzyme extracts (Frieden and Ottesen, 1959; Matthew and Parpia, 1971), L-tyrosine proved to be the best substrate for the assay of tyrosinase activity, due to enzyme specificity for this substrate. As a result of low tyrosine uptake rate by *Streptomyces* (Baumann and Kocher, 1974), *Streptomyces* tyrosinase has traditionally been assayed against L-DOPA. This substrate proved very successful for assays carried out in this study.

Spot tests had limited effectivity and appeared to be restricted to high tyrosinase concentrations. Shortcomings in the assay and spot test methods were circumvented by utilizing the zymogram method as a sensitive and rapid method for detecting tyrosinase (Goetsch, 1992). Zymogram analysis was successful at detecting enzyme presence due to highly visible colour reactions on the gel. It also differentiates between stable proteins which do not degrade on migration and are indicated by pigment deposition at specific bands on the gel, and unstable loosely-bound protein assemblies. Although these protein assemblies may oxidize the phenolic substrate, their degradation during migration is indicated by pigment formation along the solvent front. This degradation may explain the discrepancies between assay and zymogram results. Assays give an indication of total enzyme activity, while zymograms differentiate between stable and unstable protein assemblies. Zymograms were also used to determine the presence of isoenzymes. Although the response time of this method is longer than enzyme assays (3-4 hours vs.

15 minutes), zymograms appear to be the most specific method for the detection of stable tyrosinase enzyme; an important quality for application in industry. When used together, enzyme assays and zymogram analyses give both a quantitative and qualitative indication of the presence of enzyme activity.

The tyrosinase catalytic mechanism

Compared to other enzyme systems, little is known about the tyrosinase catalytic mechanism. The role of copper in the active site and the presence of a stereospecific reaction rate control site has been elucidated, and substrate and product inhibition of enzyme activity has been demonstrated. Little is known of the actual substrate and product binding and release mechanisms. This study has indicated the presence of such a mechanism by the detection of constant surges of substrates and products throughout the enzyme reaction. It is important to realize that the catalytic activity of an enzyme is a property of the whole assembly i.e. enzyme, substrate and solvent. The rate of substrate binding is dependent on factors such as diffusion of the substrate molecules in solution. In the same way diffusion may limit product dissociation from the enzyme (Suckling, 1985). In the case of tyrosinase, a bond formed between enzyme and product causes irreversible changes in the active site of the enzyme. It is therefore essential to prevent enzyme-product binding, rather than improve product diffusion away from the active site. This may be achieved by adjusting the pH of the reaction medium to make it more nucleophilic, thereby encouraging product removal by binding to the solvent. The optimization of reaction medium pH would therefore be two-fold, i.e. maintaining enzyme activity and improving enzyme stability by reducing enzyme-product binding.

The determination of phenol removal efficiency

The final stage of the screening programme was the determination of phenol removal efficiencies of enzyme extracts and whole cells in phenolic liquid media. Although not designed to replace existing methods of phenolic treatment and disposal (e.g. activated sludge methods and anaerobic methods such as landfill co-disposal), the use of enzymes and whole cells may be most successfully applied for the treatment of environmental phenol pollution, e.g. the treatment of phenolic spills.

In this study, AECI culture no. 26 intracellular enzyme extract removed 46.4% of the phenol in a simulated phenolic effluent, compared to the 72.1% by crude mushroom tyrosinase. Although there is a significant difference between removal abilities, AECI culture no. 26 presents a promising alternative for the viable production of tyrosinase for industrial use. As this is a bacterial culture, growth and enzyme yield can be optimized for the production of large quantities of enzyme in continuous fermentation systems. Whole cells in phenol-containing growth medium showed good phenol removal, confirming the efficiency of this system.

Streptomyces produced high levels of tyrosinase on assay and showed good phenol removal efficiencies in liquid media. Although no removal was seen at a 0.01% phenol level, *S. antibioticus*, *S. glaucescens* and *S. lividans* (pIJ702) showed good removal in 0.1% and 1.0% phenol-containing growth media. Though the pellets that formed in liquid media are oxygen-limiting, they aid removal and reuse of the enzyme-containing microbial biomass. The use of whole cells for phenol removal reduces enzyme inactivation and instability as the enzyme is protected within the cell.

The highest removal efficiency detected was that of *A. bisporus* mycelial cultures in liquid media. Removal efficiencies of 75.5% and 82.5% were recorded for *A. bisporus* and mushroom spawn mycelia, respectively. This study has therefore verified existing accounts of tyrosinase production by mushroom mycelia. Discrepancies noted in the literature are probably due to the variability of tyrosinase production by different cultures, as well as the essential role played by the growth medium. Disparity may also arise due to difficulties in enzyme isolation and extraction.

On evaluating the screening programme, the following procedure is recommended for future studies. Growth on phenolic agar plates remains the best preliminary indicator of enzyme presence. Zymogram analysis should then be carried out for a reliable assessment of enzyme presence, activity and stability. Finally, enzyme assays and phenol removal studies can be implemented to confirm enzyme activity and its application in industrial phenol removal.

Applications of the phenolase enzyme system

One of the aims of this project was to determine the feasibility of the polyphenolase enzyme system for industrial use. The following applications of the enzyme system give a good indication of the commercial viability of polyphenol oxidases.

1. Tyrosinase catalyzes the removal of phenolic compounds from aqueous industrial effluents.
2. Tyrosinase can be used to produce commercially valuable polyphenolic products in aqueous systems.
3. Tyrosinase produces melanin from a tyrosine substrate in aqueous systems. Melanin is an ideal material for photoprotective applications (e.g. sunscreens) as it absorbs a broad band of electromagnetic radiation. It is also useful to the cosmetic industry due to the variety of colours that are available. Melanin is generally obtained from two sources, i.e. extracted from natural materials (squid ink sacs or bovine retinas) or made synthetically from DOPA. Both methods show low melanin yields (della-Cioppa *et al.*, 1990). The use of tyrosinase for the production of large quantities of melanin in continuous fermentation systems presents a viable alternative to existing methods of melanin production.
4. Tyrosinase can be harnessed for the production of fine chemicals in organic systems e.g. L-DOPA from L-tyrosine.

This study primarily examined the application of polyphenolases for industrial effluent treatment and investigated enzyme activity in organic solvent systems. Results were promising and indicated the potentially effective use of these enzyme systems for the treatment of environmental phenolic pollutants.

Recommendations and proposals

From the results obtained in this project, the following recommendations can be proposed:

1. Although batch fermentation systems are traditionally used for enzyme production, high tyrosinase yields must be optimized in continuous culture fermentation systems. Optimization is more rapidly achieved in continuous than in batch systems as single environmental parameters can be varied independently of each other and their effects on enzyme production noted.

The use of alternative carbon and nitrogen sources such as wastes from food production and processing, e.g. cassava starch and corn steep liquor respectively, should be investigated. The use of manufacturing wastes as a nutrient source reduces the costs involved in producing a commodity by fermentation, and also reduces the residual pollution load of the waste. A problem associated with utilizing waste is its variability in composition and availability. The composition of a waste product must be carefully screened so as to exclude constituents toxic to microorganisms (Winkler, 1983).

2. The enzyme's substrate binding and release mechanism must be elucidated in order to interpret what control may be exercised over the dephenolization process.
3. Stability is an important requirement of commercial enzymes. Purified enzymes lose activity rapidly upon storage and crude enzymes are subject to inactivation by chemical, physical and biological factors, such as substrate inhibition and product binding. Although *Streptomyces* tyrosinase activities were high, enzyme instability undermines its usefulness. Enzyme stabilization by cross-linkage of enzyme molecules, bonding to carriers (immobilization) or incorporation within semi-permeable membranes (Crueger and Crueger, 1984) should be investigated. The use of detergentless microemulsions (e.g. hexane-isopropanol-water) eliminate the difficulties associated with using reversed micelles e.g. product recovery from solvents containing a high concentration of solvent (Vulfson *et al.*, 1990).

The use of whole cell systems for phenol removal eliminate problems such as enzyme inactivation due to the protection of the enzyme system within the cell.

4. Genetic and mutation selection studies should be initiated for the optimization of enzyme production by AECI culture no. 26 and the Streptomyces.
5. Enzyme extracts and whole cell systems should be tested against real industrial effluents to properly assess their phenol removal abilities.
6. This study has covered the initial stages of a typical industrial research programme, i.e. the origination of the concept, the basic laboratory research and the preliminary economic evaluations of the product. From this stage, the proposed concepts and ideas need to be evaluated in scale-up studies. Although an attempt has been made to understand and optimize conditions for biomass production and tyrosinase induction of selected microbial cultures, the subject remains intricate and sometimes unclear. Despite the complexity of the tyrosinase enzyme system, it is a valuable industrial commodity and deserves a thorough investigation, which this study has attempted to fulfil.

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APPENDIX 1 - MICROORGANISMS SELECTED FOR SCREENING AND SELECTIVE MEDIA

MEA - malt extract agar
PDA - potato dextrose agar
WA - Wickerham's agar
OA - oats agar
C/P - carrot plug agar
NA - nutrient agar
M3 - malt 3 medium
SA - Sabouraud agar

<i>Verticillium lateritium</i>	PDA
<i>Rhizopus stolonifer</i>	WA
<i>Syncephalastrum racemosum</i>	WA
<i>Thamnidium elegans</i>	WA
<i>Trypospermum myrti</i>	OA
<i>Schizophyllum commune</i>	WA
<i>Stereum hirsutum</i>	WA
<i>Chaetomium elatum</i>	PDA
<i>Sordaria fimicola</i>	C/P
<i>Alternaria alternata</i>	PDA
<i>Bacillus</i> sp.	PDA
<i>Fusarium culmorum</i>	PDA
<i>Penicillium claviforme</i>	PDA
<i>Tricothecium roseum</i>	PDA
<i>Mucor hiemalis</i>	WA
<i>Neocosmospora vasinfecta</i>	PDA
<i>Penicillium spinulosum</i>	PDA
<i>Trichoderma viridae</i>	PDA
<i>Sclerotium rolfsii</i>	PDA
<i>Microascus triganosporus</i>	OA

<i>Coriolus versicolor</i>	MEA
<i>Neurospora crassa</i>	MEA
<i>Stachybotrus chartarum</i>	MEA
<i>Coprinus comatus</i>	MEA
<i>Botrytis cinerea</i>	MEA
<i>Streptomyces antibioticus</i>	M3
<i>Streptomyces glaucescens</i>	M3
pIJ702 in <i>Streptomyces lividans</i>	M3

Environmental samples

AECI culture no. 7	NA
AECI culture no. 26	NA
AECI culture no. 29	NA
AECI culture no. 32	NA
Hogsback culture no. 1	SA
Hogsback culture no. 2	SA
Hogsback culture no. 3	SA and NA
Hogsback culture no. 4	SA
Hogsback culture no. 5	SA
Hogsback culture no. 6	SA and NA
Hogsback culture no. 7	SA and NA
<i>Suillus bovinus</i> spore culture	MEA
<i>Suillus bovinus</i> stipe culture	MEA
<i>Suillus bovinus</i> cap culture	MEA
<i>Suillus bovinus</i> gill culture	MEA
<i>Termitomyces umkowaani</i> gill culture	MEA
<i>Termitomyces umkowaani</i> stipe culture	MEA

LURGI effluent

Mushroom compost

Deep liquid culture of *Agaricus bisporus* mycelia

APPENDIX 2 - MEDIA FOR THE CULTURING OF SAMPLES

Wickerham's agar

malt extract	3g
yeast extract	3g
BACTO-peptone	5g
glucose	10g
agar	20g
distilled water up to 1 litre	

Oats agar

Rolled oats	50g
distilled water	500ml
agar	17g
distilled water	500ml

Steam oats and 500ml water for 1 hour. Mix in blender till smooth. Prepare agar with remaining distilled water and melt in water bath if necessary. Mix with oats and autoclave. Finally filter through sieve.

Carrot plug agar

Cut strips of fresh carrot. Place small plug of cotton wool in bottom of test tube. Add the carrot strip. Add distilled water to partially cover carrot strip. Autoclave.

Sabouraud broth

nutrient broth	5g
BACTO-peptone	5g
glucose	40g
distilled water up to 1 litre	

For the preparation of agar plates, add 12g.l^{-1} agar to liquid media.

APPENDIX 3 - GLUCOSE-PHENOL MINIMAL MEDIA

Media composition per litre:

glucose	5g
NH ₄ Cl	1g
K ₂ HPO ₄	1g
MgSO ₄ ·7H ₂ O	0.2g
FeSO ₄ ·7H ₂ O	0.01g
CaCl ₂ ·2H ₂ O	0.01g
CuSO ₄ ·5H ₂ O	0.025g
yeast extract	1.0g
trace element solution	0.1ml
distilled water up to 1 litre	
adjust pH to 7.2	

Pfennig's trace element solution (1 litre)

ZnSO ₄ ·7H ₂ O	0.1g
MnCl ₂ ·4H ₂ O	0.03g
H ₃ BO ₃	0.3g
CoCl ₂ ·6H ₂ O	0.2g
CaCl ₂ ·2H ₂ O	0.01g
NiCl ₂ ·H ₂ O	0.02g
Na ₂ MoO ₄ ·2H ₂ O	0.03g
FeCl ₂ ·4H ₂ O	1.5g
distilled water up to 1 litre	

Solid and liquid media

For the preparation of phenolic agar plates, add 12g.l⁻¹ agar to liquid media and phenol to the required concentration i.e. 0.01, 0.1 or 1.0%. For shaker-flask preparations, 0.05% phenol/tyrosine (1:1) is added to liquid minimal media.

APPENDIX 4 - MEDIA FOR CULTURING *Streptomyces*

Malt 3 (M3) medium

Difco Malt Extract	24g
Oxoid Yeast Extract	5g
tyrosine	0.5g
distilled water up to 1 litre	
adjust pH to 7.2	

For the preparation of M3 agar plates, add 12g.l^{-1} agar to liquid media.

Yeast extract - Malt extract (YEME) medium

Difco Yeast Extract	3g
Difco Bacto-Peptone	5g
Oxoid Malt Extract	3g
glucose	10g
sucrose	340g
distilled water up to 1 litre	
After autoclaving, add $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (2.5M)	2ml

Glucose-yeast medium

(Method taken from Gardner and Cadman, 1990)

yeast extract	10g
malt extract	10g
glucose	5g
mineral solution	1ml
dihydrogen potassium phosphate (0.05M)	6.8g
distilled water up to 1 litre	
Adjust pH to 7.2	

Add glucose and mineral solution after autoclaving. Glucose was added from a concentrated sterile solution (250 mg.ml⁻¹).

Mineral solution (per litre)

CaCl ₂ .2H ₂ O	1g
FeSO ₄ .7H ₂ O	1g
ZnSO ₄ .7H ₂ O	1g
CuSO ₄ .5H ₂ O	0.5g
MnSO ₄ .4H ₂ O	4g

Autoclave separately.

MMT medium (for tyrosinase expression and melanin production)

Minimal media (MM)

L-asparagine	0.5g
K ₂ HPO ₄	0.5g
MgSO ₄ ·7H ₂ O	0.2g
FeSO ₄ ·7H ₂ O	0.01g

Dissolve all ingredients in distilled water (final volume 1 litre), adjust pH and autoclave.
Finally add all other autoclaved solutions.

Supplement 1 litre MM with the following sterile solutions;

Casaminoacids (30%)	20ml
L-tyrosine (0.75%)	50ml
CuCl ₂ (1%)	10ml
glucose (50%)	20ml
Tiger milk	7.5ml

Tiger Milk (per 100ml)

L-arginine	1g
L-cystine	0.75g
L-histidine	0.75g
L-serine	0.75g
L-leucine	0.75g
L-phenylalanine	0.75g
L-proline	0.75g
adenine	0.15g
uracil	0.15g
nicotinamide	0.01g
make up to 100ml with distilled water	

For the preparation of agar plates, add 12g.l⁻¹ agar to liquid media.

APPENDIX 5 - THE SOMOGYI-NELSON METHOD FOR GLUCOSE DETERMINATION

Materials

Reagent A

Na_2CO_3	25g
potassium sodium tartrate	25g
NaHCO_3	20g
Na_2SO_4	200g
distilled water	1 000ml

Reagent B

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	30g
distilled water	200ml
concentrated H_2SO_4	0.4ml

Reagent C

1 part B + 25 parts A - made fresh before use.

Arsenomolybdate reagent

Solution A:	Ammonium molybdate	25g
	distilled water	450ml
	concentrated H_2SO_4	21ml
Solution B:	$\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$	3g
	distilled water	25ml

Mix solutions A and B, make up to 500ml with distilled water and incubate at 55°C for 30 minutes and store in a dark bottle.

Method

1. Prepare 0 - 0.3 g.l⁻¹ glucose standards.
2. Prepare tubes containing 1ml reagent C and 0.5ml distilled water.
3. Add 0.5ml standards and samples to above tubes.
4. Boil for 20 minutes.
5. Add 1ml arsenomolybdate reagent and mix rapidly.
6. Keep at room temperature for 10 minutes.
7. Add 9.5ml distilled water.
8. Centrifuge at 3 000 rpm for 5 minutes if necessary.
9. Read absorbances at 520nm.
10. Construct a glucose standard curve and determine sample glucose concentrations.

APPENDIX 6 - PROTEIN DETERMINATIONS

LOWRY METHOD (adapted from Lowry *et al.*, 1951)

Materials

Reagent A: Prepare 10% (w/v) Na_2CO_3 in 0.5N NaOH.

Reagent B: Prepare 1% (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in distilled water.

Reagent C: Prepare 2% (w/v) potassium tartrate in distilled water.

Reagent D: Prepare 0.03% bovine serum albumin (BSA) in distilled water.

Reagent E: 2N Folin-Ciocalteu reagent.

Method

1. Place 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 1ml BSA in test tubes. Add 0.1, 0.5 and 1ml sample to test tubes.
2. Bring the total volume of all test tubes to 1ml with distilled water.
3. Mix together 15ml reagent A, 0.75ml reagent B and 0.75ml reagent C. Add 1ml of this solution to each test tube and mix thoroughly.
4. Incubate tubes for 15 minutes at room temperature.
5. Add 5ml 2N Folin-Ciocalteu reagent to 50ml distilled water and mix thoroughly.
6. Rapidly add 3ml Folin-Ciocalteu solution to each tube and mix immediately.
7. Incubate the samples for 45 minutes. Avoid exposure to light.
8. Determine the absorbance of each sample at 660nm as soon as possible after incubation.
9. Construct a protein standard curve and determine sample protein concentrations.

BRADFORD'S PROTEIN ASSAY (Bradford, 1976)

Materials

Bradford's Protein Assay Reagent

Coomassie brilliant blue	100mg
95% ethanol	50ml
Phosphoric acid	100ml
Distilled water	made up to 1 litre

The Coomassie blue is dissolved in ethanol before addition of phosphoric acid.

Method

1. Prepare a range of BSA solutions of concentration $100\mu\text{g} - 1\,500\mu\text{g.ml}^{-1}$.
2. Prepare appropriate dilutions of all samples.
3. Pipette $100\mu\text{l}$ of each standard and sample into 5ml Bradford's Protein Assay Reagent and mix.
4. Read the absorbance at 595nm after 30 minutes.
5. Construct a protein standard curve and determine sample protein concentrations.

APPENDIX 7 - ENZYME ACTIVITY GELS (ZYMOGRAMS)

Prepare the gel casting chamber (Hoefer, SE 215) and set up the vertical slab gel unit as described in pages 8-12 of the instructions booklet for "Mighty Small" Miniature Slab Gel Electrophoresis Units (Hoefer Scientific Instruments). The 7.5% polyacrylamide resolving gel is prepared, poured and overlaid with a thin layer of saturated-isobutanol to maintain a horizontal interface between resolving and stacking gels. Once the resolving gel is set, the butanol is poured off, the gel dried with filter paper and the 3% polyacrylamide stacking gel poured. The well comb is inserted and once set, the gel sandwich is transferred to the slab gel unit. Bath buffer is poured into the upper and lower buffer chambers, samples loaded into the wells and the gel run. All buffers and substrates used are listed below.

Resolving gel buffer (1M Tris-HCl, pH 8.8)

Tris	60.6g
Conc. HCl	7.3ml
distilled water	500ml

Stacking gel buffer (1M Tris-HCl, pH 6.8)

Tris	60.6g
Conc. HCl	41.0ml
distilled water	500ml

Acrylamide stock

Acrylamide	75g
Bis-acrylamide	2g
distilled water	250ml

Bath buffer (stock, dilute 1:10)

Tris	15.2g
Glycine	72.1g
distilled water	500ml

7.5% Resolving gel

Acrylamide	7.1ml
pH 8.8 Tris buffer	10.6ml
distilled water	10.8ml
1.5% ammonium persulphate (prepared before use)	1.4ml
TEMED	7.1 μ l

3% Stacking gel

Acrylamide	1.5ml
pH 6.8 Tris buffer	1.9ml
distilled water	10.3ml
1.5% ammonium persulphate (prepared before use)	0.7ml
TEMED	20.0 μ l

Enzyme substrates

Aqueous substrates are prepared in pH 7 50mM phosphate buffer and organic substrates in chloroform at the following concentrations:

tyrosine	3.3g.l ⁻¹
L-DOPA	1.0g.l ⁻¹
phenol	4.0g.l ⁻¹
p-cresol	4.0g.l ⁻¹

APPENDIX 8 - SALT FRACTIONATION

PILOT-SCALE FRACTIONATION EXPERIMENT (Clark and Switzer, 1977)

1. Dispense 3ml samples to 8 centrifuge tubes.
2. Add solid ammonium sulphate to each tube according to the following table.

Tube number	grams salt	% saturation
1	0	0
2	0.696	40
3	0.783	45
4	0.87	50
5	0.98	55
6	1.08	60
7	1.20	65
8	1.31	70

Tube number 1 is stored on ice for later assays.

3. Add salt slowly and swirl till dissolved.
4. Stand on ice for 15 minutes.
5. Centrifuge tubes at 10 000 rpm for 15 minutes.
6. Decant the supernatant and dissolve the pellet in 3ml pH 5.4 0.03M acetate buffer.
7. Assay all samples for proteins using 0.1 and 0.2ml samples of the undilute solutions.
8. Calculate total protein (in mg) in each of the tubes. Consider total protein in tube no. 1 to be 100% and calculate protein percentages in tubes 2-8.
9. Assay all tubes for enzyme activity.

PARTIAL PURIFICATION BY SALT FRACTIONATION

(Information from table 10-1, Clark and Switzer, 1977).

1. Dispense 5ml samples into test tubes.
2. Add 1.13g (40% ammonium sulphate saturation), swirl slowly till dissolved, stand on ice for 15 minutes and centrifuge at 10 00 rpm for 15 minutes.
3. To the supernatant add 0.6g (60% ammonium sulphate saturation), dissolve, stand on ice and centrifuge as in step 1.
4. To the supernatant add 0.31g (70% ammonium sulphate saturation), dissolve, stand on ice and centrifuge as in step 1.
5. Discard the supernatant and resuspend the pellet in 3ml pH 6.0 0.1M sodium phosphate.
6. Dialyze overnight against pH 6.0 0.1M sodium phosphate.
7. Assay all samples for enzyme activity.

APPENDIX 9

Research projects initiated by this study include the following:

1. Polyphenolase enzyme systems - Honours project, 1989.
2. Mushroom polyphenol oxidase: Biomimetics and biocatalysis - Doctoral project, initiated 1990.
3. Purification of mushroom tyrosinase - Honours project, 1991.
4. Molecular studies on *Streptomyces* tyrosinase for activity in organic solvent systems - Masters project, initiated 1991.
5. The effect of organic media on the activity of mushroom tyrosinase - Honours project, 1992.